MOLECULAR MECHANISMS OF HEPATITIS C VIRUS-ASSOCIATED STEATOSIS

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Research in Partial Fulfillment of the Requirement for the Degree
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ABSTRACT

Hepatitis C virus (HCV) infects millions of people worldwide and is one of the leading causes of liver damage. Infection with HCV is strongly correlated with an increased risk of steatosis, or fatty liver disease, which is caused by a build-up of fat deposits in hepatocytes. All genotypes of HCV appear to cause some degree of steatosis in approximately 50% of infected individuals, especially in the presence of contributing host factors such as diabetes, obesity and alcoholism. However, approximately 70% of genotype 3a infections exhibit steatosis. Furthermore, successful clearance of the genotype 3a virus results in eradication of the steatosis, suggesting the genotype 3a virus may be able to directly cause steatosis.

Research suggests a role for the core protein of HCV, which forms the capsid of the virus, in the alteration of lipid metabolism pathways during infection. As such, I hypothesized that: 1) HCV alters lipid metabolism pathways and causes the build up of lipid in hepatocytes and the development of steatosis; 2) HCV-3a core protein has a differential or increased effect on these pathways in comparison to 1b core protein; and 3) other HCV proteins could also play a role in the altering of lipid metabolism.

The first study characterized the genotype 3a core protein and compared it to genotype 1b core. Very little research had been done on the 3a core protein; I showed that it localized to lipid droplets in a similar manner to genotype 1b core protein and that mutation of the same residues responsible for lipid droplet localization in 1b core also reduced lipid droplet localization in the 3a core protein. I then examined how the 1b and 3a core proteins effected the transcriptional activation of Fatty Acid Synthase (FAS), a key enzyme involved in the production of triglycerides in hepatocytes. The 3a core was

able to up-regulate transcriptional activity of FAS almost two-fold higher than 1b core. When I examined the effect of the lipid droplet localization mutants on FAS up-regulation, I found a striking difference between the two proteins. The change of a single amino acid at position 164 from a tyrosine in 1b and a phenylalanine in 3a was able to dramatically reduce the effect of 3a core on FAS up-regulation when mutated to the 1b core amino acid. This result suggested that this single amino acid may play an important role in altering lipid metabolism and causing steatosis to develop, especially in the case of genotype 3a infection.

My next step was to examine the mechanisms by which genotype 1b and 3a core up-regulated FAS. FAS transcription is mediated by a transcription factor, Sterol Response Element Binding Protein-1 (SREBP-1), which also controls the transcription of many different genes involved in lipid metabolism. Activity of SREBP-1 is controlled post-translationally by cleavage from a precursor protein to the mature protein that can act as a transcription factor. Processing of SREBP-1 is greatly increased in the presence of 3a core protein in comparison to 1b core. In addition, transcriptional activity from the Sterol Response Element (SRE), the binding recognition sequence for SREBP-1, was also greatly increased. Processing of SREBP-1 can be influenced by activity of Akt, an important cell signaling kinase. I found that 1b and 3a core proteins increased the phosphorylation of Akt, suggesting that increased activity of Akt may play a role in increasing SREBP-1 activity. When I inhibited Akt activity using a chemical inhibitor, LY294002 or Akt VIII, or a dominant negative Akt construct, the transcriptional activity from the SRE was significantly decreased in the presence of the core proteins. This study suggests that at least part of the mechanism by which the core proteins increase SREBP-1

activity occurs via increased Akt activity.

Lastly, I wanted to examine other HCV proteins for a similar effect on SREBP-1 and FAS. Very little is known about the function of HCV non-structural protein-2 (NS2) and it would be interesting to know if it could influence lipid metabolism. Interestingly, NS2 was also able to up-regulate SREBP-1 and FAS transcription via the SRE. A dominant negative mutant of SREBP-1 was able to prevent up-regulation of SREBP-1 and FAS, suggesting the direct involvement of SREBP-1 on the up-regulation of FAS and SREBP-1 by NS2. This study provides an interesting function for the NS2 protein that has not been previously shown.

The evidence provided in these studies shows a very important role for HCV in altering lipid metabolism during infection that may lead to the development of steatosis. Current research suggests that the SREBP-1 pathway may be critical in the life cycle of the virus and these studies have provided important information on how these pathways are being changed by the virus. Hopefully this work can help identify potential treatment options for HCV that can slow down disease progression by preventing the development of steatosis or by decreasing viral replication.

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DEDICATION

I would like to dedicate this thesis to my amazing, supportive and brilliant husband, Dustin. He has always been there to listen to my excitement about a new idea or to give me a shoulder to cry on after a difficult day. Without him, none of this work would ever have been completed.

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LIST OF ABBREVIATIONS

ADRP	Adipose Differentiation Related Protein
ALT	Alanine Aminotransferase
ApoAII	Apolipoprotein AII
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ARF	Alternate Reading Frame
ATP	Adenosine Triphosphate
BVDV	Bovine Viral Diarrhea Virus
C-FLIP	Cellular FLICE (FADD-like IL-1ß-converting enzyme)-Inhibitory Protein
cAMP	Cyclic Adenosine Monophosphate
CD81	Cluster of Differentiation 81
CD95	Cluster of Differentiation 95
CD95Ligand	Cluster of Differentiation 95 Ligand
cDNA	Complimentary Deoxyribonucleic Acid
CIDE-B	Cell Death-Inducing DFFA-like Effector B
CTL	Cytotoxic T Lymphocytes
DNA	Deoxyribonucleic Acid
ECMV	Encephalomyocardio Virus
EM	Electron Microscopy
ER	Endoplasmic Reticulum
FADD	Fas-associated Death Domain

FAS	Fatty Acid Synthase
GBV-B	GB Virus-B
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine Triphosphate
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Insig	Insulin-induced gene
IRES	Internal Ribosome Entry Site
IRS-1	Insulin Receptor Substrate-1
JFH-1	Japanese Fulminant Hepatitis 1
JNK	c-Jun N-terminal Kinase
KB	Kilobase
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LXR	Liver X Receptor
LXRE	Liver X Receptor Element
MAPK	Mitogen-activated Protein Kinase

MAVS	Mitochondrial Antiviral Signaling
MC	Mixed Cryoglobulinemia
MHC	Major Histocompatibility Complex
mTOR	Mammalian Target of Rapamycin
MTP	Microsomal Transfer Protein
NASH	Non-Alcoholic Steatohepatitis
NK	Natural Killer
NM	nanometer
NS2	Non-structural 2
PA28gamma	Proteosome Activator 28 gamma
PBMC	Peripheral Blood Mononuclear Cells
PDK1	Pyruvate dehydrogenase kinase, isozyme 1
PH	Plekstrin Homology
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKR	Double-stranded RNA-dependent protein kinase
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCAP	SREBP Cleavage Activating Protein
SCID	Severe Combined Immunodeficiency

Smad3	Mothers against decapentaplegic homolog 3
SOCS3	Supressor of Cytokine Signalling 3
SOCS7	Supressor of Cytokine Signalling 7
SR-BI	Scavenger receptor class B type I
SRE	Sterol Response Element
SREBP	Sterol Response Element Binding Protein
STAT3	Signal Transducer and Activator of Transcription 3
SVR	Sustained Virologic Response
TNF-R1	Tumor Necrosis Factor
TNFα	Tumor Necrosis Factor Alpha
TRAIL	TNF related apoptosis induced ligand
UTR	Untranslated Region
VAP-A	VAMP (vesicle-associated membrane protein)-associated protein A
VAP-B	VAMP (vesicle-associated membrane protein)-associated protein B
VLDL	Very Low Density Lipoprotein

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 HEPATITIS C VIRUS CHARACTERISTICS

Hepatitis C virus is a major causative agent of liver disease worldwide. An estimated 3% of the world's population are chronic carriers of the virus, including approximately 300 000 Canadians. Each year, 3 to 4 million people are newly infected with HCV, primarily through contact with infected human blood by sharing needles in intravenous drug use or by contaminated blood transfusions. Acute infection is most commonly asymptomatic but can be accompanied by fatigue and jaundice with 80% of infected individuals progressing to a chronically infected state. Chronic infection is often characterized by the development of hepatitis, steatosis, fibrosis and liver cirrhosis approximately 20 to 30 years after infection. Some patients will eventually die of HCV due to extensive liver damage, primarily due to the host immune response, or the development of hepatocellular carcinoma (HCC). Currently there is no vaccine for HCV and only 54% of HCV infected individuals respond positively to combined therapy of pegylated interferon alpha-2b plus ribavirin, depending on HCV genotypic variation and other underlying health problems. Continued HCV research is clearly necessary to develop an effective vaccine or antiviral therapy for this devastating disease.

1.1.1 HCV structure

1.1.1.1 Virus particles

HCV is an enveloped virus with a positive sense RNA genome in the *Hepacivirus* genus of the *Flaviviridae* family. Each virus particle is approximately 55-65 nm in size (Kaito et al., 1994; Shimizu et al., 1996). Electron microscopy (EM) of the virus has been hampered by the lack of a cell culture system that produces a sufficient amount of

virus for visualization. However, recent work using the JFH-1 cell culture system has allowed for further characterization of the virus (Wakita et al., 2005). By examining other viruses of the *Flaviviridae* family, it is believed that HCV has an icosahedral arrangement in which the structural glycoproteins E1 and E2 are embedded into a bi-layer lipid envelope derived from the host cells. The core protein forms the nucleocapsid of the virus that encloses the RNA genome (Ishida et al., 2001). There are three forms of the virus present in the serum of infected individuals including free virions, immunoglobulin associated virions and virions associated with very-low-density and low-density lipoproteins (Bradley et al., 1991; Thomssen et al., 1993).

1.1.1.2 Positive sense RNA genome

Hepatitis C virus contains a positive sense, single stranded RNA genome of approximately 9.5 kb. The genome consists of a single open reading frame flanked by 5' and 3' untranslated (UTR) regions which are important for replication of the genome. The highly conserved 5' UTR is 341 nucleotides long and contains the internal ribosome entry site consisting of four major RNA domains with extensive secondary structure. The entire 5' UTR is believed to be important for IRES activity and HCV translation (Beales et al., 2001; Jubin et al., 2000; Lukavsky et al., 2000). The 3' UTR is potentially important in initiating viral replication. It consists of a poly (U)/polypyrimidine tract, a variable 40 nucleotide sequence, and a highly conserved 98 nucleotide sequence with stable secondary structure (Tanaka et al., 1996; Kolykhalov et al., 1996).

1.1.1.3 HCV genotypes and quasispecies

The discovery of HCV was unique in that the existence of the virus was confirmed using molecular cloning techniques rather than direct biological methods. The

viral sequence was first obtained by extracting all nucleic acid from the serum of a non-A non-B hepatitis infected chimpanzee, creating cDNA clones and identifying the clone corresponding to the HCV genome (Choo et al., 1991). Using these methods, different strains of HCV were indentified and categorized into at least six major genotypes based on nucleic acid sequence alone. Genotypes are approximately 65% identical across the whole HCV genome. Within each genotype, subtypes are also evident with approximately 80% nucleic acid similarity (Simmonds et al., 1994). The different genotypes have different geographical distributions and prevalence, with genotype 1 being the most prevalent genotype in North America and Europe while genotype 4 is most common in Egypt and North Africa and genotypes 5 and 6 are most common in South Africa and Hong Kong. Genotypes 2 and 3 are common in North America, Europe and Japan, but to a lesser extent than genotype 1. A high number of subtypes are evident in Africa and Southeast Asia, suggesting that this region may be the original source of HCV as fewer subtypes are evident in Europe and North America (Smith and Simmonds, 1997). Currently, genotyping of the virus in an infected individual is accomplished by DNA hybridization, restriction length fragment polymorphism, direct nucleotide sequencing using polymerase chain reaction, and serologic genotyping. Many of the current methods, excluding direct nucleotide sequencing, cannot discriminate between viral subtypes.

It has become very clear that the HCV genotype is clinically relevant. In acute infection, the rate of progression to chronic infection has been related to genotype, with an increased number of genotype 1 infected individuals progressing to chronicity in comparison with other genotypes (Amoroso et al., 1998). Perhaps more importantly,

treatment response rates to combination therapy are directly correlated with HCV genotype. Genotype 1 or 4 infected individuals will only respond to treatment approximately 40-50% of the time, and only with a longer course of therapy than genotype 2 and 3 infected individuals who are able to clear the virus in approximately 70 to 80% of cases with a shorter treatment duration. The reason for this dramatic difference between genotypes is largely unknown, but may be related to an interferon sensitivity-determining region in the NS5a protein (Enomoto et al., 1995; El-Shamy et al., 2008; Torres-Puente et al., 2008).

Interestingly, HCV genotype has also been correlated with the development of steatosis in HCV infected individuals. Several studies have shown that a substantially increased number of HCV genotype 3a infected patients have steatosis, even in the absence of contributing factors such as obesity, diabetes and alcoholism. Furthermore, steatosis is abolished upon successful treatment and clearance of the virus, suggesting that HCV genotype 3a may play a direct role in altering hepatic lipid metabolism that is not as evident with other genotypes.

Within each HCV infected individual, many different sequences of the virus exist which are 90-99% identical at the nucleotide level and are members of the same infecting genotype. These populations of virus, called quasispecies, are generated by the error-prone nature of the HCV RNA-dependent RNA polymerase during genomic replication. These quasispecies populations exists in high numbers during acute infection; as infection progresses, the number of dominant quasispecies populations decreases with the major quasispecies strain changing intermittently. The role of these quasispecies populations is two fold. Firstly, high mutation rates can result in the development of new viral strains

that have increased viral fitness over the parent strain. Secondly, viral quasispecies likely serve as a mechanism for immune evasion and viral persistence. As the immune system mounts an antibody response against a particular viral epitope, the virus quickly mutates and the dominant viral population changes to one that the immune system no longer recognizes. In a similar fashion, the virus can also quickly generate mutants that are resistant to antiviral drugs. The high mutability of the HCV virus poses several challenges to the development of an effective vaccine or antiviral drug.

1.1.2 HCV proteins

The HCV genome is translated into a polyprotein that is subsequently cleaved into approximately 10 proteins in the sequence Core-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b. The Core, E1, E2 and p7 proteins are considered structural proteins of the virus while NS2, NS3, NS4a, NS4b, NS5a and NS5b are non-structural proteins.

1.1.2.1 Structural proteins

The core protein is cleaved from the polyprotein via host signal peptidase to form a 191 amino acid immature form of the protein. The core protein is then cleaved between amino acids 173 to 179 by host Signal Peptide Peptidase (SPP) to form the mature protein (Okamoto et al., 2004). The core protein is a dimeric alpha-helical protein that can bind RNA and consists of two major domains. The D1 domain is primarily hydrophilic and is located in the N-terminus while the D2 domain is primarily hydrophobic and located in the C-terminus. D2 assists in the folding of the protein as well as membrane and lipid droplet association (Boulant et al., 2005; Santolini et al., 1994). The core protein is localized on the endoplasmic reticulum and on the surface of lipid droplets in infected cells. Some studies have also shown a small amount of core protein in the nucleus and

on mitochondria, but this localization has not been observed in infected cells (McLauchlan, 2000; Hope et al., 2002; Suzuki et al., 2005; Rouille et al., 2006). Localization of core with lipid droplets is also mediated by proteolytic cleavage by SPP (McLauchlan et al., 2002). Mature core proteins form the nucleocapsid of the virus. However, many studies have shown alternative roles for the core protein in the pathogenesis of HCV including apoptosis, insulin resistance, cell cycle, and lipid metabolism. Interestingly, the core protein-encoding region also contains a +1 alternative reading frame that results in translation of the ARF (alternative reading frame) protein. The F protein does not contain an AUG start codon, so translation of this protein is a random event. No known function of the F protein is currently known. Although antibodies against it are present in HCV infected individuals, it is not required for viral replication (Branch et al., 2005).

The E1 and E2 proteins of HCV are envelope glycoproteins that are essential for virus entry and form heterodimers on the viral envelope. The E2 protein contains a hypervariable region that mutates frequently to allow for the development of immune escape variants (Forns et al., 2000; Callens et al., 2005).

The p7 protein has recently been found to have ion-channel activity and it is essential for virus infectivity. As such, new research has been directed toward developing antiviral compounds to block the function of this protein (Saint et al., 2009).

1.1.2.2 Non-structural proteins

The NS2 protein is a hydrophobic protease that cleaves the HCV polyprotein between NS2 and NS3 and it may function as a novel cysteine protease. Efficient cleavage of NS2/3 is required for HCV replication, but the presence of NS2 is not

required for genome replication. Following cleavage, NS2 is localized in the ER membrane. Besides its protease activity, NS2 appears to be involved in apoptosis, cell proliferation, innate immunity and lipid metabolism (Dumoulin et al., 2003; Erdtmann et al., 2003; Yang et al., 2006; Oem et al., 2007).

The NS3 protein is a protease and RNA helicase. Protease activity of NS3 is mediated by the NS4a protein. Both proteins are localized on the ER. The NS3/4a protease complex cleaves the HCV polyprotein downstream of NS3 and this action is essential for formation of the viral replication complex (Lindenbach et al., 2005). NS3/4a is able to inhibit the host innate immune response via disrupting the RNA helicase retinoic acid-inducible gene-I (RIG-I) pathway by cleaving Cardif, an adaptor protein of interferon regulatory factor-3 (Foy et al., 2003). The function of the NS3/4a helicase activity is still unknown.

The NS4b protein is present on the ER membrane and induces intracellular membrane changes that may be involved in the formation of the replication complex membranous webs (Hugle et al., 2001; Egger et al., 2002). It also contains a nucleotide binding motif that hydrolyzes GTP (Einav et al., 2004). NS4b is thought to play a major role in viral replication, assembly and release as well as lipid metabolism (Jones et al., 2009; Park et al., 2009).

NS5a is also an ER-associated protein that is particularly multi-functional. It can bind to HCV RNA and it is essential for viral replication (Huang et al., 2005). It can be hyperphosphorylated in order to inhibit viral replication (Evans et al., 2004). NS5a is also able to decrease the immune response via inhibition of interferon-induced double stranded RNA activated protein kinase PKR (Gale et al., 1998). NS5a may also play a

role in mediating lipid metabolism as it interacts with core proteins on the surface of lipid droplets and it also interacts with apolipoproteins (Shi et al., 2002).

NS5b is an RNA dependent RNA polymerase that synthesizes RNA using an RNA template. It is also localized on the ER membrane and it is essential for viral replication. As such, it is a prime target for the development of antiviral compounds (Lohmann et al., 2000).

1.1.3 HCV life cycle

1.1.3.1 Receptors and entry

The entry of HCV into a naïve host cell appears to be a complicated, multi-step process involving several different host receptors. The initial entry steps have only been recently characterized thanks to the newly developed infectious cell culture system, and many details are still to be discovered. Heparin, a glycosaminoglycan, has been long suspected to play a role in viral entry with several studies showing inhibition of viral attachment in the presence of heparinase, an enzyme that disrupts heparin (Koutsoudakis et al., 2006; Barth et al., 2003; Barth et al., 2006). Low-density lipoprotein receptor (LDLR) is very likely a critical component of viral attachment. HCV in the serum of infected patients is bound to low density and very low-density lipoproteins. Adsorption of HCV from the serum of infected patients can be inhibited through the use of antibodies against LDLR suggesting that the association of HCV with lipoproteins is an important step in attachment of the virus (Thomssen et al., 1992; Andre et al., 2002; Huang et al., 2007a; Monazahian et al., 1999; Molina et al., 2007). CD81, a cell surface protein in the tetraspanin family, is considered one of the major players in HCV attachment. Antibodies against CD81 effectively inhibit HCV infectivity in different models. In

addition, HepG2 and HH29 hepatoma cells, which are not normally permissive to HCV infection, can be infected following ectopic expression of CD81 (Cormier et al., 2004; Wakita et al., 2005. However, expression of CD81 in other non-permissive cell lines does not allow HCV infection to occur, suggesting that other host cell factors may be involved in the attachment and entry process (Bartosch et al., 2003). Scavenger receptor class B type I (SR-BI) is another cell surface protein that may be involved in viral attachment and entry. SR-BI is a receptor for lipoproteins and it can also change the lipid composition of membranes. Blocking of SR-BI with antibodies can prevent virus entry. It is still unclear whether SR-BI is interacting directly with the virus proteins or if it is interacting with virus associated lipoproteins to mediate virus entry as both mechanisms have been observed (Bartosch et al., 2003; Catanese et al., 2007; Acton et al., 1996; Maillard et al., 2006; Huang et al., 2003). Recently, two tight junction cell proteins Claudin-1 and Occludin have been shown to be involved in HCV entry. Claudin-1 can be ectopically expressed in non-permissive, non-hepatic 293T cells and allow HCV infection to occur. Knock-down of Claudin-1 in permissive Huh7 cells can reduce HCV infectivity. It has also been found that Claudin-1 and Occludin are involved in late entry of the virus into the cell, which likely occurs through interaction with the tight junctions (Evans et al., 2007). The use of the tight junctions by HCV may also allow for cell to cell spread of the virus (Timpe et al., 2008).

It has been shown that HCV entry is mediated by endocytosis in a pH-dependant manner. In addition, knock-down of clathrin, a critical component of the endocytotic vesicles, prevents HCV infection (Meertens et al., 2006; Blanchard et al., 2006). Fusion

of the virus envelope with the endosome membrane is not well characterized and both E1 and E2 are likely involved in the process (Lavillette et al., 2007).

1.1.3.2 Replication, assembly and release

HCV replication occurs in a membrane associated replication complex that contains several viral and host proteins and replicating RNA. The positive strand of the virus RNA genome is made into a negative strand that is then used to make more positive strand copies for packaging. The NS5b protein is the major player in the synthesis of both the positive and negative strands. The HCV replication complex appears to contain a membrane alteration called a membranous web that can be observed by electron microscopy in infected cells *in vitro* and *in vivo*, as well as in cells expressing the NS4b protein alone. The source of the membranous web is likely the ER as most of the HCV proteins are associated with the ER upon translation (Shi et al., 2003; Gosert et al., 2003). It is also possible that viral replication is occurring on lipid rafts that contain large amounts of cholesterol and sphingolipids. Inhibitors of sphingolipid synthesis can prevent HCV replication and both non-structural proteins and replicating RNA are found on lipid rafts (Sakamoto et al., 2005; Matto et al., 2004; Aizaki et al., 2004; Shi et al., 2003). Recently, it has been determined that lipid droplets play an important role in virus assembly. Core protein localizes around lipid droplets where it recruits non-structural proteins and replication complexes. Inhibiting lipid droplet localization of core protein decreased the production of infectious virus particles (Miyanari et al., 2007). There are also many different host cell proteins with putative roles in HCV replication. For example, vesicle-associated membrane protein-associated proteins A and B (VAP-A and VAP-B) localize in the ER and bind both NS5a and NS5b and are essential for viral

replication (Hamamoto et al., 2005). Cyclophilin B, a peptidyl-prolyl cis-trans isomerase, binds to NS5b and enhances its RNA binding activity (Watashi et al., 2005). Similarly, p68, and RNA helicase, binds NS5b and moves into the cytoplasm from the nucleus to assist in viral replication (Goh et al., 2004).

Very little is known about HCV assembly and release. There are several different forms of the virus found in infected serum, including free mature virions, virions bound to low-density and very low-density lipoproteins, virions bound to immunoglobulins and non-enveloped nucleocapsids. It is believed that viral RNA can interact with the core protein, which then oligomerizes to form the nucleocapsid around the viral RNA (Tanaka et al., 2000). The envelope of the virus is then obtained through core interaction with E1 glycoprotein on the ER membrane, from which budding occurs (Ezelle et al., 2002; Murakami et al., 2006). From this point it is thought that the virus is released from the cell after transit through the Golgi apparatus and the secretory pathway (Serafino et al., 2003).

1.1.3.3 Virus-like particles

In the serum of HCV infected individuals, several different forms of HCV can be found that differ in both size and density. Currently very little is known about HCV assembly and how these different types of HCV particles are formed and secreted. HCV RNA can be found in infected serum at a density between 1.03 and 1.25 g/ml (Thomssen et al., 1992; Thomssen et al., 1993). Interestingly, the lower density particles appear to be more infectious than the higher density particles when tested in chimpanzees (Bradley et al., 1991; Hijikata et al., 1993). The low-density particles contain high amounts of triglycerides, as well as HCV RNA, core protein and apolipoproteins B and E (ApoB and

ApoE). Triglycerides along with ApoB and ApoE are the major components of very-low density lipoproteins (VLDL) and low-density lipoproteins (LDL) which are particles packaged in the ER of hepatocytes to be secreted into the bloodstream and transport their triglyceride load to adipocytes for storage (Rustaeus et al., 1999). During HCV infection, assembled virus particles are hypothesized to use this pathway to acquire ApoB, ApoE and triglycerides prior to secretion from the cells. Virus particles that do not obtain the necessary triglycerides and apolipoproteins are not secreted from the cell and undergo degradation. Exploitation of the VLDL assembly pathway by HCV ultimately decreases lipid export from the cells and could be an important factor in the development of steatosis in infected patients. The addition of apolipoproteins and triglyceride to the virus particle could also be advantageous to the virus as it could be providing a mechanism of immune escape by hiding viral epitopes and also by increasing the number of potential receptors (ie. CD81, LDLR and SR-BI) for attachment (Gastaminza et al., 2008; Huang et al., 2007a; Nielsen et al., 2006; Andre et al., 2002; Perlemuter et al., 2002).

Another fraction of the HCV population in infected serum appears to be non-enveloped. These particles have a higher density and vary in size. The core protein is displayed on the surface of these particles and antibodies against core protein are a hallmark of HCV infection. Currently it is unknown whether these nonenveloped particles are infectious or what role they play in the course of HCV infection and pathogenesis (Maillard et al., 2001).

1.1.4 HCV molecular pathogenesis

1.1.4.1 Cell survival and apoptosis

Apoptosis is a very important mechanism for the removal of virus infected cells and many viruses inhibit apoptotic pathways in order to ensure their continued survival. However, in chronic HCV infection an increased amount of apoptosis is observed in the liver although it is unknown whether HCV infected or uninfected hepatocytes are undergoing apoptosis (Calabrese et al., 2000; Bantel et al., 2001). Three major death ligands, CD95 Ligand, Tumor Necrosis Factor α (TNFα) and Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL), are considered to be the major players in inducing hepatocyte apoptosis. Infection with HCV up-regulates all three of these death ligands. In addition, CD95 Ligand induced apoptosis was observed in uninfected bystander cells of HCV infected livers (Calabrese et al., 2000; Ghavami et al., 2005; Ando et al., 1997; Gremion et al., 2004). Interestingly, TRAIL can also induce the development of steatosis (Mundt et al., 2005). When specific HCV proteins were examined for their influence on apoptosis, results showed that all HCV proteins have both pro-apoptotic and anti-apoptotic effects depending on the model being used and it is currently unclear as to what occurs *in vivo.* Core proteins have been shown to inhibit TNFα and CD95 Ligand induced apoptosis in cell culture, while other studies have shown that the presence of core protein, including those expressed in transgenic mice, cannot inhibit CD95 Ligand induced apoptosis (Ray et al., 1998; Ruggieri et al., 1997; Machida et al., 2001). Core proteins can directly interact with several pro-apoptoic proteins including CD95, TNF-R1 and lymphotoxin-β (Zhu et al., 1998; Matsumoto et al., 1997). However, it can also bind to FADD, C-FLIP and Smad3 to prevent apoptosis

(Saito et al., 2006; Pavio et al., 2005). Core can also increase phosphorylation of Akt, an important protein involved in cell survival (Waris et al., 2007). Core proteins also induce oxidative stress that can lead to increased susceptibility to apoptosis (Okuda et al., 2002; Machida et al., 2006). The E1 protein inhibits TRAIL-induced apoptosis, while E2 can induce caspase dependent apoptosis (Lee et al., 2007; Chiou et al., 2006). NS2 can inhibit CIDE-B induced apoptosis, while NS3 can cleave Cardif and prevent RIG-I associated apoptosis (Erdtmann et al., 2003; Meylan et al., 2005). NS4a can induce caspase-8 independent apoptosis from the mitochondria (Nomura-Takigawa et al., 2006). NS5a has shown both anti-apoptotic and pro-apoptotic effects. It can inhibit Bax-mediated apoptosis by acting as a bcl-2 homologue. It can also activate the PI3K/Akt pathway and prevent p53- mediated apoptosis (Chung et al., 2003; Lan et al., 2002; Street et al., 2004). However, direct apoptosis has been observed in cells expressing NS5a (Macdonald et al., 2004). All of these results suggest an important but complex role of HCV and its proteins in mediating cell survival and apoptosis that is yet to be fully explained. The anti-apoptotic potential of this virus may influence the development of hepatocellular carcinoma, a common complication of HCV infection (Balsano and Alisi, 2007).

1.1.4.2 Immunopathogenesis

It is generally accepted that liver damage during HCV infection is primarily caused by the reaction of the immune system to virus infection, although some cytopathic effects of the virus such as increased apoptosis and the development of steatosis have been observed. HCV itself has also been shown to alter both the innate and adaptive immune responses. The NS3/4a protease is able to induce cleavage of mitochondrial

antiviral signaling protein (MAVS), which results in the impairment of RIG-I mediated interferon production in the presence of dsRNA (Meylan et al., 2005). During viral infection, cytotoxic lymphocytes are critical to the efficient removal of infected cells. In acute HCV infection, the appearance of HCV specific cytotoxic $CD8^+$ T cells (CTLs) is associated with decreased viremia (Shoukry et al., 2003). Activation of HCV specific $CD38^+$ $CD69^+$ T cells coincides with inflammation and hepatitis during acute infection, suggesting a role for these non-interferon γ producing CTLs in causing immune mediated liver damage (Lechner et al., 2000). Regulatory T cells have been shown to have an important role in suppressing HCV-specific CTL responses, as increased numbers of regulatory T cells are present in chronically infected patients versus those who have spontaneously recovered from their infection (Cabrera et al., 2004). Therefore, it is likely that CTLs play a role in both controlling viremia and causing liver damage during HCV infection.

Natural Killer (NK) cells are also important lymphocytes involved in clearing viral infection. NK cells can directly or indirectly induce apoptosis in virally infected hepatocytes that may contribute to both viral clearance and liver damage. HCV infected cells have decreased MHC class I protein expression, which is a primary inducer of NK mediated cell lysis. Conversely, NK cells may actually protect the liver from progression to fibrosis by removing activated hepatic stellate cells, the main fibrogenic cell type in the damaged liver. Indeed, increased NK cytolytic activity is inversely correlated with the fibrosis stage in the liver (Morishima et al., 2006).

γδ T cells are also important lymphocytes in the liver during viral infection. Increased levels of γδ T cells are present in the livers of chronic HCV patients (Tseng et

al., 2001). However, γδ T cells have also been shown to have a protective effect on the liver during chronic viral infection, suggesting that they too may also decrease liver injury during infection (Fu et al., 1994).

1.1.4.3 Lipid metabolism

One of the major functions of the liver is to metabolize lipids. As such, infection of hepatocytes with HCV is likely to affect cellular pathways involved in lipid metabolism. Indeed, patients infected with HCV show clinical disease associated with lipid metabolism defects such as steatosis and hypolipoproteinemia (Serfaty et al., 2001). *In vitro* studies have also demonstrated an important role for the virus in exploiting lipid metabolism pathways in order to produce virus particles and evade the immune system that contribute to HCV pathogenesis (Ye, 2007). These pathways will be discussed in further detail in section 1.4.

1.2 HCV CLINICAL CHARACTERISTICS

1.2.1 Acute viral infection

Upon infection with HCV, most patients enter an acute phase that is largely asymptomatic. Most of the infected individuals (~80%) will progress to chronic HCV infection while the other 20% will clear the virus within the first 3 months after infection. The asymptomatic nature of the acute stage of infection results in difficulty in diagnosing and reporting HCV infection, as well as difficulty in studying the aspects of the immune response that allow for viral clearance during this time. The greatest risk of infection with HCV is through intravenous drug use, unprotected sex, exposure during medical procedures, vertical transmission from mother to child, or needle injury in health care professionals. Blood transfusion prior to 1992 is also an important risk factor (Centers

for Disease Control and Prevention. Surveillance for acute viral hepatitis United States, 2005. 56th ed. Atlanta, GA: Centers for Disease Control and Prevention; 2007. P. 1-24). HCV RNA can be detected in the serum approximately 1-3 weeks after exposure. Symptoms include fatigue, jaundice, dyspepsia, and abdominal pain but are generally not specific or severe enough to warrant consultation of a doctor. Elevated alanine aminotransferase (ALT) levels can be detected 4-12 weeks after exposure and are the first indication of liver injury (Santantonio et al., 2003). Serum HCV RNA and anti-HCV antibody seroconversion is generally used to detect HCV infection (Pawlotsky, 2002). Host and viral factors appear to contribute to viral clearance in acute infection including genotype of the virus, HIV co-infection, gender, race, age and HLA (Lehmann et al., 2004). A strong cellular immune response is also thought to increase viral clearance, and symptomatic infection appears to correlate with viral clearance (Gruner et al., 2000; Thimme et al., 2001). Patients who spontaneously clear the virus need to be monitored as disease relapse may occur up to 6 months after clearance (Gerlach et al., 1999).

1.2.2 Chronic viral infection

Infection with HCV is one of the leading causes of liver disease worldwide. As previously discussed, a small proportion of people are able to clear the virus following an acute infection but the majority progress to a chronically infected state. Chronic infection can persist for many years without causing the death of the patient. However, most cases eventually lead to the development of hepatitis, fibrosis, steatosis and cirrhosis with the eventual need for a liver transplant if left untreated. A small number of HCV infected individuals will also develop hepatocellular carcinoma. Host factors such as age, obesity, alcohol use, gender, race and co-infection with HIV or HBV can increase the rate of

progression of liver damage (Soto et al., 1997; Pontisso et al., 1998; Corrao and Arico, 1998). Genotype does not appear to play a role in disease progression (Silini et al., 1996). However, the presence of non-alcoholic steatohepatitis can increase progression of disease, and infection with genotype 3a is associated with a higher prevalence of steatosis (Mihm et al., 1997; Adinolfi et al., 2001). In general, the rate of progression to cirrhosis is unpredictable in chronic HCV infection (Ascione et al., 2007).

1.2.3 Associated clinical pathology

1.2.3.1 Hepatitis, steatosis, fibrosis and cirrhosis

As previously discussed, HCV is not generally considered to be a cytopathic virus. Damage to the liver is thought to be primarily the result of immunopathogenic mechanisms. However, HCV has been associated with direct cytopathic effects on the liver such as apoptosis, stellate cell activation, the development of steatosis and insulin resistance. Damage to the liver is clinically observed by increased ALT levels. In the case of HCV, liver biopsies are often taken to assess the stage of liver disease. Hepatitis of the liver occurs following an infiltration of inflammatory cells into the liver and damage of the hepatocytes leads to steatosis and fibrosis. Steatosis of the liver is the accumulation of lipid within hepatocytes that can eventually lead to the development of fibrosis. Fibrosis of the liver is characterized by the accumulation of extracellular matrix proteins, like collagen, that are produced by different liver cell types following damage. Liver fibrosis can be reversed (Bataller and Brenner, 2005). Cirrhosis is the final stage of liver disease in which liver tissue is replaced by scar tissue and nodules that decrease liver function. Cirrhosis is irreversible and patients in the advanced stages of liver cirrhosis will need a liver transplant (Mengshol et al., 2007).

1.2.3.2 Lymphoproliferative disorders

One of the best characterized extra-hepatic disorders associated with HCV infection is mixed cryoglobulinemia (MC). MC is caused by the development of cryoglobulins consisting of polyclonal IgG and monoclonal IgM or polyclonal IgM with rheumatoid factor that precipitate in the blood at less than 37°C (Zignego 1997). Symptoms include weakness, arthralgias, and palpable purpura in the lower extremities and complications can include kidney damage and possibly liver damage (Ferri 1992; Kayali 2002; Saadoun 2006). Approximately 5-10% of HCV patients exhibit MC (Lunel 1994). Successful treatment of the HCV infection results in eradication of MC in most patients (Zignego 2007b). HCV is also associated with malignant lymphomas that may be related to MC. B-cell derived Non-Hodgkin's Lymphoma is the most common lymphoma associated with HCV infection (De Vita 1997).

1.2.3.3 Hepatocellular carcinoma

Infection with HCV is a major risk factor for the development of hepatocellular carcinoma (HCC). The mechanism by which HCV causes HCC is unknown but several studies have indicated a role for the core protein, including the induction of oxidative stress and steatosis. Cellular signaling pathways are also changed by the core protein, as previously discussed. The development of liver fibrosis and cirrhosis also increases the risk for HCC. Approximately 1-3% of HCV patients will develop HCC after 30 years of chronic infection (Hassan et al., 2002). Age, alcohol consumption, obesity, diabetes, genotype and co-infection with HBV or HIV can all increase the risk of developing HCC in HCV infected patients (El-Serag, 2002).

1.2.4 Treatment

1.2.4.1 Ribavirin and interferon combination therapy

The current standard of treatment for HCV is a combination therapy of pegylated interferon α with ribavirin. Treatment is generally successful in eradicating the virus in approximately 50% of genotype 1 patients and 80% of genotype 2 and 3 patients. Treatment duration is 48 weeks in genotype 1 infection and only 24 weeks or even less in genotype 2 and 3 infection. Sustained Virological Response (SVR) is determined by undetectable HCV RNA levels 24 weeks after the end of treatment; the large majority of patients who achieve SVR remain virus free and have reduced liver damage (Zeuzem, 2008). Interferon α is a non-specific antiviral agent that acts by inducing 2', 5' – oligoadenylate synthetase, double stranded RNA activated protein kinase and myxovirus proteins. It may also indirectly enhance the function of NK cells, CTLs and macrophages (Chevaliez and Pawlotsky, 2007).

The mechanism of action of ribavirin is largely unknown. Studies have shown a potential involvement in modulating the immune response, inhibiting inosine monophosphate dehydrogenase, directly inhibiting HCV NS5B, inducing mutagenesis, and modulating interferon-stimulated gene expression (Hofmann et al., 2008). New protocols for treatment doses and duration are constantly being updated.

1.2.4.2 Anti-viral drugs

There are currently several HCV-specific antivirals going through clinical trials. Antiviral drugs are being developed to target the NS3/4a protease; these antivirals include telaprivir and boceprivir. In phase 3 clinical trials both of these antivirals can increase

Sustained Virological Response (SVR) rates in genotype 1 patients when used in combination with interferon α and ribavirin (Dusheiko et al., 2008; Schering Plough http://www.schering-plough.com/Schering_plough/news/release.jsp?releaseID=1182855). Monotherapy with these antivrals is unlikely due to the rapid development of resistant mutants following treatment. R1626, a nucleoside inhibitor of the HCV NS5b, has been shown to reduce viral load when used in combination with interferon α and ribavirin (Roberts et al., 2008). It is likely that future treatment for HCV will include antivirals in combination with standard interferon α/ ribavirin therapy and that this strategy will significantly increase SVR rates.

1.2.4.3 Vaccines

Vaccine development for HCV has been hampered by several issues. Currently, the role of neutralizing antibodies, the hallmark of most vaccines, in clearing HCV infection is undetermined largely due to the limited ability to grow the virus in cell culture and also the highly variable genome of the virus. However, it is clear that helper and cytotoxic T cell responses play an important role in viral clearance. Keeping this in mind, several avenues are being pursued for an effective HCV vaccine candidate. These include: 1) a vaccine that will prevent infection (ie. prophylactic); 2) a vaccine that would enhance viral clearance following infection; or 3) a vaccine that would induce viral clearance when given during chronic infection (ie. therapeutic). Several different vaccine candidates are currently in clinical trials although none have been approved for use (Strickland et al., 2008).

1.3 HCV EXPERIMENTAL MODELS

1.3.1 Replicon cells

Up until 1999, one of the only methods available to examine HCV pathogenesis and infection *in vitro* was by transiently transfecting HCV genes, alone or in combination, into different cell types. This method is straightforward and effective and allows for the examination of the effects of a single HCV protein on a cell. However, transient transfection of DNA is not the most accurate model to study the effects of an RNA virus *in vitro*. Unfortunately, attempts at establishing a robust HCV cell culture system had been unsuccessful and so the various aspects of the HCV life cycle could not be examined. As such, the development of the HCV replicon system by Lohmann *et al.* provided a new and important model to study HCV pathogenesis and replication (Lohmann et al., 1999).

Using previous protocols established with Bovine Viral Diarrhea Virus (BVDV) and Kunjin, two members of the Flaviviridae family, a subgenomic molecular clone of genotype 1b HCV was developed that contained the 5'UTR, a neomycin resistance cassette, the ECMV IRES and the HCV NS3 to 3'UTR region. Once transcribed into RNA, the subgenomic replicon construct was electroporated into Huh7 human hepatoma cells and stably transfected cells were selected using G418. Expression of HCV proteins and replication of the HCV subgenome was confirmed (Lohmann et al., 1999). This system has since been used to develop genomic replicons that, for as yet unknown reasons, do not assemble and produce infectious virus particles (Ikeda et al., 2002). Further optimization of this procedure has allowed it to become a very critical component of HCV research, although some aspects of the HCV life cycle such as entry and

assembly cannot be studied. In addition, Huh7 cells appear to be the only cell line to permit high levels of replication of the replicon, although other cell lines can support low levels of replication (Uprichard et al., 2006; Ali et al., 2004; Zhu et al., 2003). Another interesting aspect of the replicon system is that certain regions of the replicon subgenome mutate in order to adapt to cell culture and replicate efficiently, and these mutations could partially explain the difficulty in infecting cells in culture with a wild type strain (Lohmann et al., 2001; Krieger et al., 2001).

1.3.2 JFH-1 infectious cell culture system

The most important development in HCV research in recent years has been the discovery of the JFH-1 strain. The JFH-1 virus was isolated from a patient with fulminant hepatitis and identified as genotype 2a, although with some novel mutations. Kato *et al*. cloned the JFH-1 virus into a subgenomic replicon and examined its replication in several different cell lines. Surprisingly, the subgenomic JFH-1 replicon replicated at a much higher level than other previously utilized replicons. In addition, JFH-1 could also replicate in cell lines other than Huh-7 and no adaptive mutations appeared in the replicon expressing cell lines (Kato et al., 2001; Kato et al., 2003). To further examine JFH-1, a full-length genomic construct was made. Following electroporation of the full-length JFH-1 construct, Huh7 cells were able to produce infectious virus particles, although at a low titre. These particles could infect naïve Huh7 cells as well as a chimpanzee, proving its validity as an experimental model (Wakita et al., 2005; Zhong et al., 2005). Subsequent work by many groups have improved the JFH-1 cell culture system by creating chimeric viruses with other HCV genotypes or viruses with mutations that improve viral replication and particle production (Kato et al., 2007;

Yi et al., 2006; Scheel et al., 2008; Gottwein et al., 2007). The HCV cell culture system will continue to provide a wealth of information on the HCV life cycle.

1.3.3 Animal models

1.3.3.1 Chimpanzees

Chimpanzees are the most important animal model available for HCV research. No other animal can be successfully infected with HCV and develop a disease that closely resembles that of human HCV infection. In chimpanzees, HCV infection proceeds in much the same way as in humans, with many animals developing a chronic infection, although symptoms are milder. Acute infection can also be studied in chimpanzees as this is often difficult in humans due to the non-specific nature of the symptoms. Immune responses to HCV can also be assessed and more liver tissue can be obtained for detailed molecular studies than is possible in humans. Chimpanzees could also play an important role in the testing of vaccine candidates. Despite this, chimpanzee studies are limited due to cost and ethical considerations. Small study numbers (usually 2-4 animals) hamper statistical analysis and ultimate conclusions of the experiments. As such new animal models are desired for further *in vivo* HCV research (Bukh, 2004; Walker, 1997; Kolykhalov et al., 1997; Bigger et al., 2001; Bukh et al., 2001b).

1.3.3.2 Chimeric mice

One of the most innovative animal models for HCV research was developed in 2001 by Mercer *et al.* Mice with Severe Combined Immunodeficiency (SCID) were engineered to contain a plasminogen activator transgene. These mice then had human hepatocytes injected into their livers. The human hepatocytes were successfully transplanted into the mouse liver. These mice can then be infected with HCV-positive

human serum and support viral replication in the transplanted human hepatocytes (Mercer et al., 2001). Although these mice cannot be used for most work related to the immune response, they have been used to examine the pathogenesis of HCV and as a model for various HCV antiviral compound trials (Hsu et al., 2003; Kneteman et al., 2009; Joyce et al., 2009; Inoue et al., 2007; Walters et al., 2006).

1.3.3.3 Other animal models

Another commonly used model for HCV research is transgenic mice. Transgenic mice that express single HCV proteins, or a combination of HCV proteins, have been developed. These mice have been used to show pathogenesis associated with expression of the HCV proteins (Koike et al., 1995; Pasquinelli et al., 1997; Kawamura et al., 1997; Wakita et al., 1998). For example, Moriya *et al.* demonstrated that HCV Core expressing mice developed both steatosis and hepatocellular carcinoma (Moriya et al., 1997; Moriya et al., 1998). Transgenic mice can provide a lot of information on HCV pathogenesis, but they cannot be used for virus life cycle studies or in clinical trials for HCV vaccines or antivirals, and as such their use is limited.

Another proposed animal model for HCV is the use of GB Virus B (GBV-B) as a surrogate model in tamarins. GBV-B is the most closely related virus to HCV and it is only known to infect tamarins, marmosets, and owl monkeys. However, GBV-V is most commonly associated with acute infection and rarely results in chronic infection. Despite this, further research will be needed to determine if chimeric HCV/GBV-B viruses could be made that could infect tamarins and establish a chronic infection similar to HCV in humans. This model could be more effectively used for antiviral and vaccine studies (Muerhoff et al., 1995; Bukh et al., 2001a; Warter et al., 2009; Akari et al., 2009).

1.4 INFLUENCE OF HCV ON LIPID METABOLISM

1.4.1 Clinical evidence

1.4.1.1 Blood lipid and cholesterol

A well-known feature of chronic HCV is the presence of hypolipoproteinemia. Decreased apolipoprotein B serum levels in chronic HCV patients are correlated with the degree of steatosis and fibrosis. Genotype 3a infection in particular is associated with a more pronounced hypobetalipoproteinemia than other genotypes. Following successful treatment and eradication of the virus, genotype 3a infected patients exhibit normal lipoprotein levels, while other genotypes do not (Moriya et al., 2003; Petit et al., 2003; Siagris et al., 2006). Furthermore, chronic HCV infection is also associated with low serum cholesterol levels. Hypocholesterolaemia is increased in genotype 3a infected patients and unlike other genotypes, it can be reversed following successful response to therapy (Fernandez-Rodriguez et al., 2006; Siagris et al., 2006). This suggests that the genotype 3a virus may cause hypolipoproteinemia and hypocholesterolaemia directly by using a different mechanism than other genotypes. Further study has also revealed a correlation between high cholesterol and low-density lipoprotein levels in the serum and successful response to therapy, thereby underlining the importance of dyslipidemia in HCV infection (Gopal et al., 2006; Akuta et al., 2007). In contrast, a recent study has examined the potential of statins in the treatment of HCV. Statins are used in the treatment of hypercholersterolaemia by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase). *In vitro* testing revealed a decreased HCV replication level in the presence of flavustatin, atorvastatin, and simvastatin. Subsequent work with HCV infected patients showed decreased viral load upon treatment with flavustatin and

strongly suggests that it could be beneficial if added to standard interferon therapy. In contrast, atorvastatin appeared to have no effect (Akuta et al., 2007; Bader et al., 2008).

1.4.1.2 Obesity

Recent evidence suggests that obesity may play an important role in treatment response and progression of disease in chronic HCV patients. Obesity has been found to be a risk factor for the development of steatosis and fibrosis in chronic HCV (Hu et al., 2004). In addition, patients with a high body mass index have a lower chance of successfully responding to therapy (Bressler et al., 2003).

1.4.1.3 Insulin resistance

Insulin plays a vital role in lipid metabolism, especially lipogenesis. Type II diabetes is a common problem in chronic HCV patients (Mehta et al., 2000; Allison et al., 1994). Insulin resistance is a precursor to the development of Type II diabetes and insulin resistance can increase the rate of progression to fibrosis in chronic HCV (Hui et al., 2003). Insulin resistance occurs when higher than normal insulin concentrations are needed to maintain a balance of metabolic responses, such as gluconeogenesis and lipogenesis. Homeostasis model assessment of insulin resistance (HOMA-IR) is used as a method of assessing insulin resistance. Patients with chronic HCV consistently have a higher HOMA-IR level than healthy controls; however genotype 3 infected patients have a lower HOMA-IR than other genotypes. Successful eradication of HCV also causes a decrease in HOMA-IR (Hui et al., 2003; Bugianesi et al., 2006; Romero-Gomez, 2006a). Insulin resistance is closely associated with the development of steatosis. It is believed that increased insulin causes increased lipogenesis in the liver; alternatively, the accumulation of fat in the liver may cause insulin resistance to develop (Adams and

Angulo, 2005; Romero-Gomez, 2006b). In addition, chronic HCV patients have high Tumor Necrosis Factor Alpha (TNFα) levels. TNFα is known to induce insulin resistance by interfering with insulin receptor substrates 1 and 2 signalling (Svegliati-Baroni et al., 1999; Aytug et al., 2003).

1.4.1.4 Steatosis

Steatosis, or fatty liver disease, is present in approximately 40-86% of chronic HCV patients of all genotypes. Only 20-30% of the uninfected population has steatosis, indicating that HCV may directly or indirectly cause the accumulation of fat in the liver resulting in steatosis. Steatosis is usually caused by obesity, diabetes or alcoholism and it can be a precursor for more severe liver disease such as non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. The prevalence of steatosis in chronic HCV is also higher than other liver diseases, suggesting that liver damage during infection is not the only reason for this association (Mihm et al., 1997; Rubbia-Brandt et al., 2000). In chronic HCV, macrovesicular steatosis is observed in the periportal region of the liver whereas in non-alcoholic steatosis in uninfected individuals steatosis is observed in the centrilobular region of the liver, suggesting that HCV induced steatosis may occur via a different mechanism (El-Zayadi, 2008).

The prevalence and degree of steatosis severity is significantly increased in people with genotype 3 infection (Rubbia-Brandt et al., 2000). When patients with genotype 3 successfully eradicate the virus after therapy, steatosis is often reversed. This effect is generally not observed in non-genotype 3 patients and they often have steatosis in conjunction with other factors such as diabetes and obesity. Steatosis is often observed in genotype 3 patients who are not obese (Patton et al., 2004; Poynard et al., 2003; Kumar

et al., 2002). This information suggests that, although all HCV genotypes contribute to the development of steatosis during chronic HCV, genotype 3 appears to have a different mechanism that may be cytopathic to hepatocytes.

1.4.2 Molecular evidence

1.4.2.1 Mechanisms of steatosis

Non-alcoholic fatty liver disease (NAFLD) is an obesity-related disorder that is characterized by the presence of steatosis. Steatosis accompanied by fibrosis and cell death is referred to as NASH. The mechanisms responsible for development of NAFLD, and specifically steatosis, are not clearly understood but are mainly associated with increased fatty acid uptake by the liver as well as *de novo* lipogenesis (Greenfield et al., 2008).

Steatosis is characterized by the accumulation of trigylcerides in liver cells. Triglycerides are neutral lipids that are composed of a glycerol backbone with three long chain fatty acids. Normally triglycerides are exported for storage in adipocytes, but in steatosis, triglycerides remain in hepatocytes for long periods of time and can promote progression to NASH, fibrosis and cirrhosis. Free Fatty Acids (FFAs) that make up triglycerides are usually obtained from the diet or from lipolysis of triglyceride stores in adipocytes. During insulin resistance, a common contributing factor to the development of steatosis, increased lipolysis of triglycerides in adipocytes occurs, providing increased FFAs for delivery to the liver. In addition, insulin resistance induces hyperinsulinemia that increases insulin binding and signaling in hepatocytes. Increased insulin induces SREBP-1c activity and, subsequently, the increased expression of genes related to fatty acid synthesis, such as FAS (Blasiole et al., 2007).

1.4.2.2 Lipid droplet association and triglyceride accumulation

One of the first indications that HCV could alter lipid metabolism was the discovery that the core protein localized to lipid droplets *in vitro* in transfected cell cultures and *in vivo* in chimpanzees infected with HCV (Barba et al., 1997). Lipid droplets are pockets of neutral lipid surrounded by a phospholipid membrane and are present in almost every type of cell. The functions of lipid droplets include lipid storage, metabolism and trafficking, membrane biogenesis, and possibly cell signaling and protein degradation (Fujimoto et al., 2008). HCV core protein was also found to co-localize with Apolipoprotein AII, a protein commonly associated with lipid droplets (Barba et al., 1997; Sabile et al., 1999). Analysis of the core protein revealed a unique, central, hydrophobic domain II that could play a role in lipid droplet association (Hope and McLauchlan, 2000). Further mutagenesis of the domain II region and comparison with the GBV-B capsid protein, that also localizes to lipid droplets, revealed two regions of the protein that were similar to lipid droplet localizing plant oleosin proteins. These sequences consisted of two proline residues at amino acid position 138 and 143, as well as a YATG sequence motif at amino acid location 164-167. Deletion or mutation of these sequences abolished lipid droplet localization (Hope et al., 2002). Comparison of different genotypes of the core protein, specifically genotype 3, showed that genotype 3 core localizes to lipid droplets as well and that the YATG sequence (FATG in 3a core) may play a role in lipid metabolism differences in genotype 3 infection (see Chapter 3) (Hourioux et al., 2007; Jackel-Cram et al., 2007). The presence of the core protein also induces larger lipid droplets to form in cells; in particular genotype 3a core proteins that are associated with steatosis can induce larger lipid droplet accumulation than core

proteins that are not associated with steatosis in chronic HCV patients (Piodi et al., 2008; Abid et al., 2005). Specific sequences of the 3a core protein have also been identified as being involved in steatosis and lipid accumulation in cells in cell culture (Jhaveri et al., 2008).

Profiling of the protein composition of lipid droplets in core-expressing cells revealed differences in lipid droplet composition that may indicate a role for core protein in lipid droplet biogenesis as well as RNA metabolism on lipid droplets (Sato et al., 2006). Core protein was found to be able to redistribute lipid droplets in cells by lowering adipocyte differentiation related protein (ADRP). This redistribution is dependent on microtubule networks and blockage of this process decreased viral production, indicating a possible role for these networks in viral assembly or release (Boulant et al., 2008).

Localization of the core protein on lipid droplets has also been found to be essential for infectious virus production. When the core protein is mutated to prevent lipid droplet localization, significantly decreased levels of infectious virus are produced in the JFH-1 cell culture system (Boulant et al., 2007). The core protein was also found to recruit HCV non-structural proteins and HCV replication complexes to the surface of lipid droplets. This recruitment process is essential for virus production, indicating that assembly of virus particles may take place close to or on lipid droplet surfaces (Miyanari et al., 2007). Indeed, further study showed that HCV assembly occurs on membranes adjacent to lipid droplets but not actually on lipid droplet membranes (Roingeard et al., 2008).

Taken together, these studies reveal the emerging importance for the lipid droplet in the HCV life cycle. Core protein localization with lipid droplets in particular plays a

very important part in this process and may also serve to explain some of the alterations in lipid metabolism observed during HCV infection.

1.4.2.3 VLDL secretion

It is well known that HCV associates with very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) in infected patients (Prince et al., 1996; Andre et al., 2002). This association may be very beneficial for the virus as it allows for some degree of immune escape and entry into cells via different host receptors (Maillard et al., 2006). The association of HCV with VLDL appears to occur by binding to sequences in the E1 and E2 proteins of the virus (Monazahian et al., 2000). Indeed, it appears as though HCV uses the VLDL/LDL assembly and secretion pathway for its own assembly and secretion. Membrane vesicles with replicating HCV contain ApoB, ApoE and Microsomal Transfer Protein, all of which are vital components of VLDL secretion. Assembly of HCV occurs in ER and post-ER compartments. Subsequent inhibition of VLDL assembly reduces HCV production and immature particles are degraded via the proteasomal pathway (Huang et al., 2007b; Gastaminza et al., 2008). Several studies have suggested that novel inhibitors of the VLDL pathway could be used as a potential treatment for HCV. These include a grapefruit flavanoid naringenin and an inhibitor of long chain acyl-CoA synthetase 3-mediated phosphatidylcholine synthesis (Yao et al., 2008; Nahmias et al., 2008). HCV core protein is known to inhibit MTP activity and decrease VLDL secretion by itself, suggesting that it may be a key mediator of the VLDL pathway during infection (Perlemeuter et al., 2002).

Levels of LDL in patient serum can also be an indicator of potential response to therapy (Akuta et al., 2007; Gopal et al., 2006). Genetic polymorphisms in the ApoB

promoter region can indicate enhanced or decreased viral susceptibility (Zhu et al., 2009). Polymorphisms in the MTP gene region are known to cause genotype 3 infected patients to have increased fat accumulation in their liver, indicating potential host involvement in the development of steatosis in genotype 3 infected individuals. (Zampino et al., 2008) These results suggest a very important role for VLDL assembly and secretion in the assembly and secretion of HCV that could provide novel therapeutic targets and partially explain the association of HCV infection with the development of steatosis.

1.4.2.4 Insulin resistance

Knowledge of the molecular mechanisms of how HCV can cause insulin resistance is incomplete. Aytug *et al* in 2003 was able to show that Insulin Receptor Substrate-1 (IRS-1) and PI3K signaling was impaired in chronic HCV liver biopsy samples (Aytug et al., 2003). Further research revealed that both IRS-1 and IRS-2 were down-regulated by HCV due to an up-regulation of Suppressor of Cytokine Signalling-3 (SOCS3). Non-response to therapy could also be correlated with an up-regulation of SOCS3 (Kawaguchi et al., 2004; Walsh et al., 2006). The core protein is primarily responsible for the effect on IRS signaling. Both genotype 1b and 3a core can down-regulate IRS-1, but use different mechanisms. Genotype 3a core down-regulates IRS-1 via PPAR gamma activation and up-regulation of SOCS7 whereas genotype 1b core activates mammalian target of rapamycin (mTOR) to cause the down-regulation of IRS-1 (Pazienza et al., 2007). Core protein is also able to increase serine phosphorylation of IRS-1 and impair Akt/PKB signaling, thereby causing decreased glucose uptake by hepatocytes (Banerjee et al., 2008). Transgenic mice expressing core protein also have

impaired ability to lower plasma glucose levels upon insulin stimulation and proteasome activator PA28 gamma is essential for the development of insulin resistance in these mice (Miyamoto et al., 2007).

1.4.2.5 Oxidative stress

Oxidative stress occurs when the body cannot remove or repair the damage caused by reactive oxygen species. Many diseases cause oxidative stress that results in some or all of the disease pathogenesis. HCV has long been known to cause oxidative stress and it may contribute to the pathogenesis of the disease, especially liver damage and hepatocellular carcinoma.

Early research showed increased Mn-superoxide dismutase production, which protects cells from oxidative stress, in the PBMCs of chronic HCV patients but not in the liver (Larrea et al., 1998). Reduced glutathione was also found in erythrocytes, suggesting oxidative stress was occurring in HCV patients (Swietek and Juszczyk, 1997). Thioredoxin, which is made in response to oxidative stress, is increased in the serum of chronic HCV patients and correlates with the degree of liver fibrosis and non-response to therapy (Sumida et al., 2000). Cells expressing the HCV replicon showed increased oxidative stress during HCV replication via the p38 MAPK and JNK signaling pathways (Qadri et al., 2004). Replicon cells also showed down-regulated gastrointestinal glutathione peroxidase that caused the cells to be more susceptible to oxidative stress (Morbitzer and Herget, 2005). Increased activation of STAT3 was also observed in replicon cells due to oxidative stress (Waris and Siddiqui, 2005). Core protein and the non-structural proteins appear to induce different antioxidant defense mechanisms (Abdalla et al., 2005; Garcia-Mediavilla et al., 2005). Core protein specifically can

induce reactive oxygen species production, lipid peroxidation products, and antioxidant gene expression by causing mitochondrial injury (Okuda et al., 2002). Core protein is known to associate with mitochondria and inhibit electron transport, thereby causing an increase in ROS production (Korenaga et al., 2005). Transgenic mice expressing the core protein often develop hepatocellular carcinoma and an altered oxidant/antioxidant state in the livers of these mice, in the absence of inflammation, was thought to contribute to this (Moriya et al., 2001). Induction of oxidative stress by the core protein can also cause activation of the PI3K/Akt pathway that subsequently increases cleavage and activation of SREBP to influence lipid metabolism (Waris et al., 2007).

High levels of oxidative stress during HCV infection can also impair interferon signaling and predict non-response to therapy (Di Bona et al., 2006). Levels of oxidative stress are also correlated with insulin resistance (Mitsuyoshi et al., 2008). Furthermore, in non-genotype 3 patients, oxidative stress and insulin resistance appear to contribute to the development of steatosis. Oxidative stress appears to play an important role in the pathogenesis of HCV and may also be involved in the alteration of lipid metabolism by HCV, but further research is needed to confirm this.

1.4.2.6 SREBP transcription factors

Sterol Response Element Binding Proteins (SREBPs) are key regulators of fatty acid synthesis in liver cells. SREBP-1a, SREBP-1c and SREBP-2 are members of the helix loop helix basic leucine zipper protein transcription factors that are controlled by lipid levels in hepatocytes. SREBPs are first made as a precursor protein that is attached to the endoplasmic reticulum membrane. At low lipid levels, SREBP is transported from the ER to the Golgi by SREBP Cleavage Activating Protein (SCAP) where it is processed

into the mature, nuclear form of SREBP and transported into the nucleus by Importin-β. Nuclear SREBP can then activate the transcription of genes containing an SRE binding element, such as Fatty Acid Synthase and Low Density Lipoprotein Receptor. Nuclear SREBP is very unstable and is quickly phosphorylated and ubiquitinated. Conversely, during high cellular lipid concentrations, Insig-1 and Insig-2 hold SCAP and the SCAP-SREBP complex remains on the ER membrane and does not get moved to the Golgi for processing. SREBP-1c is also regulated by insulin by increasing activity of PI3K/Akt and inducing changes in abundance of Insig-1 and Insig-2. Insulin causes increased transcription of SREBP-1c as well as increased proteolytic processing (Ribaux and Iynedjian, 2003; Yabe et al., 2003). Activation of SREBPs has been proposed as a direct or indirect mechanism leading to the development of steatosis (Horton et al., 2003; Shimomura et al., 1999).

The first evidence linking HCV to the activation of SREBP was discovered using microarray analysis of HCV infected chimpanzee livers. Several genes that are induced by SREBP were up-regulated in the chimpanzee livers, including FAS, ATP citrate lyase and Lipase A. Further analysis using replicon cell lines confirmed that inhibition of the fatty acid synthesis pathway using cerulenin and 25-hydroxycholesterol decreased HCV replication, suggesting that this pathway is important in the virus life cycle (Su et al., 2002). In addition, SREBP-2 regulates the transcription of genes involved in cholesterol biosynthesis including those responsible for the production of mevalonate, a critical precursor involved in lipid anchoring (Sakura et al., 2001). Inhibition of the mevalonate pathway by lovastatin can disrupt the HCV replication complex and inhibit HCV replication (Ye et al., 2003). A geranylgeranylated protein involved in mevalonate

synthesis, FBL2 is able to bind NS5a and is essential for HCV replication (Wang et al., 2005). Up-regulation of FAS was also observed in the presence of the core protein by our laboratory (see Chapter 3) and this effect was mediated by SREBP-1 (Jackel-Cram et al., 2007). Transgenic mice expressing core protein also have up-regulated FAS and SREBP-1 promoter activity; this activity is dependant on the presence of PA28 gamma. Microarray analysis of core transgenic mice with steatosis also revealed increased activity of the SREBP pathway (Moriishi et al., 2007; Chang et al., 2008). HCV core was also demonstrated to increase proteolytic cleavage and phosphorylation of SREBP-1 and -2 during cell culture infection with HCV. This effect was caused by increased oxidative stress and activation of the PI3K/Akt signaling pathway (Waris et al., 2007). Inhibition of FAS activity was also shown to significantly decrease HCV production in cell culture, suggesting this pathway may be a novel therapeutic target (Yang et al., 2008).

Core protein is not the only protein involved in activating the SREBP pathway. Our results showed that NS2 is also capable of inducing transcription of FAS and SREBP-1 (see Chapter 5) (Oem et al., 2008). NS4b has also been shown to increase transcription of SREBPs as well as protein levels of SREBP and FAS via increased PI3K/Akt activity. NS4b was also able to induce lipid accumulation (Park et al., 2009).

1.4.2.7 Akt

Akt/ PKB is a critical regulator of cellular signaling. It plays a major role in cell survival, proliferation, growth and metabolism. Akt is a serine/threonine protein kinase that is part of the cAMP-dependent protein kinase A/G/C family of kinases. These kinases are important in cancer and diabetes and share similar mechanisms of activation as well as similar structures in their catalytic domain. Three isoforms of Akt exist:

Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ. All isoforms have an amino terminal pleckstrin homology (PH) domain, a central kinase domain and a hydrophobic motif on the carboxy domain. The PH domain interacts with phosphatidylinositol (3,4,5) triphosphate (PIP3) that is a product of phosphatidylinositol 3 kinase (PI3K) activation. The central kinase domain of Akt contains a conserved Threonine residue (Thr308) which can be phosphorylated and partially regulate Akt activity. The carboxy terminal contains the hydrophobic motif F-X-X-F/Y-S/T-Y/F that is critical for Akt activation through phosphorylation of the Serine or Threonine residues (Song et al., 2005; Manning and Cantley, 2007).

Activation of Akt is mediated by growth factor signaling of receptor tyrosine kinases. These kinases activate PI3K that phosphorylates PIP2 to form PIP3 (which can be reversed by PTEN). PIP3 recruits Akt and phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane. Upon Akt binding PIP3 on its PH domain, the conformation of Akt is changed. This allows PDK1 to phosphorylate Thr308 and mTOR complex 2 phosphorylates Ser473. These residues are in slightly different positions in Akt2 and Akt3. In addition, several reports have shown phosphorylation of tyrosine residues Tyr315, Tyr326 and Tyr474 are necessary for fully activating Akt. Other research has suggested PI3K independent mechanisms of Akt activation that include activation through PKA, Ca2+/calmodulin-dependent kinase or cellular stress and heat shock. Proteins can bind to Akt and regulate its activity (Song et al., 2005; Manning and Cantley, 2007).

Akt also plays a major role in the regulation of cellular metabolism. Akt can phosphorylate and inhibit glycogen synthase kinase 3 Beta (GSK3β) in the presence of

high levels of insulin. Once GSK3β is inhibited, glucose is metabolized and stored as glycogen. Interestingly, inhibition of GSK3β may also prevent apoptosis, suggesting that the role of Akt in cell survival and metabolism may be connected (Manning and Cantley, 2007). GSK3β is able to promote degradation of SREBPs. Through inhibition of GSK3β, Akt is able to stabilize SREBPs and enhance lipid production in hepatic cells (Du et al., 2006; Porstmann et al., 2005; Fleischmann and Iynedjian, 2000; Matsumoto et al., 2002; Ono et al., 2003; Taniguchi et al., 2006).

The effect of HCV on Akt and the PI3K signaling pathway varies depending on the study and the protein involved. NS5a can interact with Grb2 to activate PI3K/Akt; it can also bind to the p85 subunit of PI3K to activate Akt (He et al., 2002; Street et al., 2004). The effect of NS5a on PI3K/Akt leads to the phosphorylation and deactivation of GSK3β. Core protein has been shown to both impair and activate PI3K/Akt signaling. In T cells, core can bind to gC1qR and result in impairment of Akt phosphorylation (Yao et al., 2004). Core also impaired IRS-1 and PI3K/Akt phosphorylation in the presence of insulin (Kawaguchi et al., 2004). Stimulation with insulin in transgenic mice expressing core also resulted in decreased Akt phosphorylation (Miyamoto et al., 2007). Despite these results, core was shown to increase PI3K/Akt phosphorylation in HCV cell culture systems in two separate studies (Waris et al., 2007; Banerjee et al., 2008). Replicon expressing cells were also able to activate PI3K/Akt by increasing production of nRas. Inhibition of nRas production or inhibition of Akt phosphorylation resulted in decreased HCV replication (Mannova and Beretta, 2005). NS4b was also able to activate PI3K/Akt by itself (Park et al., 2009).

These results suggest that the effect of HCV on PI3K/Akt is complex and may depend on the model system used as well as the cell type and the cell culture conditions employed. Nevertheless, the PI3K/Akt pathway appears to play an important role in HCV pathogenesis.

2.0 HYPOTHESIS AND OBJECTIVES

Evidence in the literature supports a strong correlation between infection with HCV and the development of steatosis, even in the absence of factors such as diabetes, obesity and alcoholism. However, clinical evidence had revealed that genotype 3a virus has a stronger association with the development of steatosis. Research had focused primarily on the genotype 1b virus and the role of the core and NS5a proteins in lipid metabolism pathways. The mechanisms for the development of steatosis during HCV infection were still unclear and very little research had examined the effect of genotype 3a virus on lipid metabolism.

As such, I hypothesized that:

1. HCV alters lipid metabolism pathways in infected hepatocytes resulting in increased lipid levels and the development of steatosis.

2. Genotype 3a virus has a stronger or differential effect on lipid metabolism pathways in comparison to genotype 1b virus.

3. Core proteins mediate the effect on lipid metabolism but other HCV proteins may also have an effect.

My objectives were:

1. (a) To clone and express the genotype 3a core protein in Huh-7 cells and compare its effect on lipid metabolism to genotype 1b core.

(b) To examine the effect of the genotype 1b and 3a core proteins on Fatty Acid Synthase, a key enzyme involved in the production of triglycerides in hepatocytes.

2. To examine the effect of the genotype 1b and 3a core proteins on Sterol Response Element Binding Protein-1, a transcription factor that controls the expression of many different genes involved in lipid metabolism in hepatocytes and determine the mechanisms of this effect.

3. To examine the effects of other HCV proteins on FAS and SREBP-1.

3.0 UP-REGULATION OF FATTY ACID SYNTHASE PROMOTER BY HEPATITIS C VIRUS CORE PROTEIN: GENOTYPE 3A CORE HAS A STRONGER EFFECT THAN GENOTYPE 1B CORE

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3.1 Abstract

Hepatitis C virus genotype-3a (HCV-3a) is directly linked to steatosis development. We studied the effects of HCV-3a core protein on the promoter activity of fatty acid synthase (FAS), a major enzyme involved in *de novo* lipid synthesis.

HCV-3a and -1b core genes were cloned and expressed. Using a FAS promoter-luciferase reporter, we demonstrated that both HCV-3a and -1b core proteins up-regulated the FAS promoter. However, HCV-3a core protein expression induced significantly higher FAS promoter activity than HCV-1b core. We further showed that FAS up-regulation by HCV core was dependent on transcription factor sterol response element binding protein-1. Mutational analysis showed that processing of HCV core protein of different genotypes was differentially involved in FAS promoter up-regulation. Although lipid droplet localization of HCV core protein was not important for FAS up-regulation, a specific amino acid residue (Phe^{164}) within the FATG lipid droplet localization sequence of HCV-3a core protein played a major role in the stronger FAS activation by HCV-3a core.

The stronger effect of HCV-3a core protein on FAS activation in comparison to HCV-1b core could contribute to the higher prevalence and severity of steatosis in HCV-3a infections.

Keywords: Hepatitis C virus, genotype 3a, core, fatty acid synthase

3.2 Introduction

Hepatitis C virus (HCV) is classified into six genotypes (Pawlotsky et al., 2003). HCV of different genotypes may cause different pathology. For example, in HCV-3a patients, the incidence of steatosis is ~70%, which is significantly higher than infections by other HCV genotypes. Furthermore, HCV-3a patients who clear the viral infection after interferon therapy have reduced levels of steatosis, while patients with other genotypes do not (Kumar et al., 2002; Poynard et al., 2003). Therefore, HCV-3a has been suggested to be a strong steatogenic factor (Kumar et al., 2002; Adinolfi et al. 2001; Hezode et al., 2004; Hwang et al., 2001; Rubbia-Brandt et al., 2001; Rubbia-Brandt et al., 2000; Sharma et al., 2004).

HCV is a positive-sense RNA virus encoding a single polyprotein, which is cleaved by host and virus-encoded proteases to generate structural and non-structural proteins (Poynard et al., 2003; Choo et al., 1991). Core protein is a structural protein, but it also modulates host cellular pathways, including those involved in lipid metabolism (Ray et al., 2001). The core protein exists in immature and mature forms. After cleavage from the polyprotein, the immature form is further processed by an endoplasmic reticulum membrane-associated signal peptide peptidase around amino acid residues 173 to 186 to generate the mature core protein (Ray et al., 2001; Kato et al., 2003; Liu et al., 1997; Lemberg et al., 2002).

HCV core protein localizes on lipid droplets (Barba et al., 1997; Shi et al., 2002; Rouille et al., 2006; Perlemuter et al., 2002), suggesting a potentially functional role in lipid metabolism. HCV-1 core protein has been shown to modulate the activity of microsomal triglyceride transfer protein, RXRalpha, and peroxisome proliferators-

activated receptor α (Perlemuter et al., 2002; Yamaguchi et al., 2005; Tsutsumi et al., 2002; Cheng et al., 2005). It is noteworthy that HCV-3a core induces increased amounts of triglyceride (TG) accumulation over the core proteins of other genotypes (Abid et al., 2005). Therefore, HCV core protein is actively involved in lipid metabolism and, more importantly, HCV-3a core may have a more profound impact than core proteins of other genotypes.

Fatty acid synthase (FAS) plays a central role in *de novo* lipid synthesis and TG accumulation in hepatocytes by catalyzing the reaction of acetyl-CoA and malonyl-CoA into palmitate which is esterfied into TG (Semenkovich et al., 1997). FAS expression is primarily regulated at the transcriptional level by sterol response element binding protein-1 (SREBP-1) (Semenkovich et al., 1997; Shimano et al., 2001). Alteration of the SREBP-1-FAS pathway can result in clinical manifestations such as steatosis and diabetes (Horton et al., 2002).

In this study, we examined the effect of HCV core protein on FAS promoter activity. We found that while the expression of HCV-3a and HCV-1b core proteins in Huh-7 cells up-regulated FAS promoter in a SREBP-1-dependent manner, the effect of HCV-3a core was significantly stronger than that of HCV-1b core. We further showed that a single amino acid residue (F^{164}) of HCV-3a core protein was critical for this process.

3.3 Materials and Methods

3.3.1 Plasmid constructs

HCV-3a infected human sera samples were used for RNA extraction by TriZol (Invitrogen). RNA was then reverse transcribed into cDNA with random hexamers and

reverse transcriptase (SuperScript III-RT, Invitrogen). HCV core gene was amplified from cDNA using core-specific primers (Forward: 5'-*aatt*gaattcATGAGCACACTTCCTAAACCTC-3', *Eco*RI site; and Reverse: 5'-*aatt*ctaga*tta*GGCATCGACCCCTGAGAACAT-3', *Xba*I site). A stop codon was incorporated in the reverse primer to terminate the translation. PCR was performed using DeepVent DNA polymerase (New England Biolabs) (35 cycles; 94ºC 30 sec, 50ºC 30 sec, 72ºC 1 min). The PCR product of 573 bps in size was cloned into the pEF/cyto/myc vector (Invitrogen) by restriction enzymes *Eco*RI and *Xba*I. HCV-1b core was cloned by a similar PCR-based approach using plasmid pDM22, a cDNA clone of HCV-BK (Manabe et al., 1994). Amino acid substitutions or deletions were achieved by primer-driven mutagenesis. All the plasmids were confirmed by DNA sequencing.

3.3.2 Cell culture, transfection, and immunofluorescence

Human hepatoma Huh-7 cells were maintained in Dulbecco's Modified Eagle's Medium (10% fetal bovine serum, 1% non-essential amino acids, 1% gentamicin) at 5% CO_2, 37ºC. Huh-7 cells grown in 4-well chamber slides were transfected with 1.0 μg of plasmid DNA using the calcium phosphate precipitation method (Graham et al., 1973). Forty-eight hours after transfection, cells were washed in PBS and fixed in 4% paraformaldehyde. Cells were then permeabilized in 0.1% Triton X-100, washed and blocked with 3% BSA in PBS. After further washing, cells were incubated with an anti-core monoclonal antibody followed by a secondary antibody (AlexaFluor-488 goat anti-mouse, Molecular Probes). Cells were then rinsed in 60% isopropanol and incubated with Oil Red O stain (dissolve 0.5 mg/mL of oil red O in isopropanol overnight, filter and

mix 6 parts oil red O to 4 parts water just prior to staining). Cells were rinsed twice in 60% isopropanol and then in PBS. DAPI (4',6-diamidino-2-phenylindole, 300 nM in PBS, Molecular Probes) was added to the cells for 5 min. After washing, the slides were mounted in Prolong Antifade (Molecular Probes) and allowed to dry overnight in the dark. For endoplasmic reticulum (ER) staining, cells were incubated with a calnexin-specific antibody (Santa Cruz Biotechnology) followed by a secondary antibody (AlexaFluor-594 goat anti-rabbit, Molecular Probes). Images were collected using a confocal microscope (Zeiss LSM410) with appropriate laser settings and processed using Adobe-Photoshop-8.0.

3.3.3 Western blot analysis

Huh-7 cells grown in 6-well plates were transfected with 2 μg plasmid DNA. Cells were lysed in lysis buffer (1% SDS, 10 mM Tris-HCl pH 8.0) 48 hours after transfection. Proteins separated in 15% polyacrylamide gels were transferred onto PVDF membranes and blocked in 2% skim milk. Membranes were incubated at 4ºC overnight with a core-specific monoclonal antibody or β-actin-specific polyclonal antibody (Cell Signaling Technology). After washing, membranes were then incubated with HRP-conjugated anti-mouse or anti-rabbit IgG antibody (Santa Cruz Biotechnology). Protein bands visualized by the Immobilon Chemiluminescence System (Millipore) were subjected to densitometry analysis using the Quantity-One software (Bio-Rad).

3.3.4 Luciferase Assays

Huh-7 cells grown in 24-well plates were co-transfected with 0.5 μg of core-expressing plasmids, 0.5 μg of a plasmid encoding firefly luciferase under the control of wild-type or SRE site deleted FAS promoter (Swinnen et al., 1997), and 0.02 μg of pRL-SV40 encoding Renilla luciferase (rLuc) (Promega). In another series of experiments, a plasmid encoding a dominant negative (DN) mutant SREBP-1 protein was included in the co-transfection. The DN-SREBP-1 gene (Heemers et al., 2001) was cloned into pcDNA3.1(+) (Invitrogen) by restriction enzymes *Eco*RI and *Spe*I. Luciferase assay was performed 48 hours after transfection with Dual Luciferase Assay reagents (Promega) using a TD 20/20 luminometer (Turner Designs). The luciferase readings of each sample were first normalized against the rLuc levels and then normalized against the corresponding amount of HCV core proteins.

3.3.5 Statistical analysis

All experiments were performed at least three times. Results were analyzed for statistical differences using Student *t* test. A p value of ≤ 0.05 was considered statistically significant.

3.4 Results

3.4.1 Cloning and expression of HCV-3a core

HCV-1b and -3a core gene was cloned into the pEF/cyto/myc vector as described in Materials and Methods. Amino acid sequence analysis showed that the 3a core protein sequence that we cloned had 98% similarity to that of a published 3a core (GenBank Accession No. D17763) and ~ 90% similarity to that of 1b (BK) core protein (Fig.1a).

Western blot analysis showed that 3a core protein was expressed at a lower level than 1b core when they were analyzed in one protein gel, although the same amount of proteins was used for loading as demonstrated by equal β-actin levels (Fig. 3.1b).

3.4.2 HCV-3a core protein localizes on lipid droplets in Huh-7 cells

As previous studies have demonstrated that HCV core protein localizes on lipid droplets (Barba et al., 1997; Shi et al., 2002; Rouille et al., 2006), we studied subcellular localization of HCV-3a core protein. Immunofluorescence experiment demonstrated that 3a core protein was found in surrounding large circular structures, which could be stained with Oil Red O, an intracellular lipid droplet stain (McVean et al., 1965) (Fig. 3.1c). In addition, co-staining of core and calnexin was observed, consistent with ER localization of HCV core protein (Fig. 3.1d).

3.4.3 HCV core protein up-regulates FAS promoter in an SREBP-1 dependant manner

Since FAS is a major enzyme in lipid metabolism, we investigated whether the expression of HCV core proteins has any effect on FAS transcription using a FAS promoter-luciferase reporter containing the 178 bp fragment of the FAS proximal promoter (Swinnen et al., 1997). Expression of 3a and 1b core proteins resulted in significantly higher luciferase activity than vector transfection (Fig. 3.2a; 3a core vs. control, p=0.00000006; 1b core vs. control, p=0.0234). Importantly, cells transfected by 3a core-expressing plasmid exhibited significantly higher luciferase activity than 1b core transfection (Fig. 3.2a, 3a core vs. 1b core, p=0.000002).

Since SREBP-1 is the major transcriptional factor for modulating FAS transcription by binding to the SRE site on the FAS promoter (Shimano et al., 2001), we studied the role of SREBP-1 in HCV core-induced FAS promoter up-regulation. For this purpose, a FAS promoter-luciferase reporter with the SREBP binding site deleted (from -63 to -46) (ΔSRE) was utilized. After transfection of the FAS-ΔSRE promoter reporter, luciferase activity was barely detectable (Fig. 3.2a), indicating that FAS up-regulation by HCV core was mediated through SREBP-1. Interestingly, the basal FAS promoter level was also suppressed after transfection of the FAS-ΔSRE promoter reporter (Fig. 3.2a), suggesting that FAS activation was mediated primarily by SREBP-1 in Huh-7 cells.

To further confirm the involvement of SREBP-1 in FAS up-regulation by HCV core proteins, Huh-7 cells were transfected with a plasmid expressing DN-SREBP-1, together with HCV core-expressing plasmids, and the wild-type FAS promoter reporter. While plasmid vector transfection did not have any effect, luciferase activity was decreased to similar levels in DN-SREBP-1 transfected cells (Fig. 3.2b).

3.4.4 Role of core protein processing in FAS up-regulation

Previous studies have shown that amino acid residues 180, 183, and 184 are involved in core protein processing (McLauchlan et al., 2002; Okamoto et al., 2004). To investigate the role of core protein processing in FAS up-regulation, a mutant carrying triple mutations (A180V, S183L, and C184V) was generated for both HCV-1b and -3a core (Fig. 3.3a). Western blot analysis showed inhibition of processing of the mutant core proteins, although processed core was still evident (Fig. 3.3b). Subcellular

localization studies of the mutant core proteins showed a reduced lipid droplet localization of both 1b and 3a core mutants (Fig.3.3c).

The processing mutants of HCV core were then used to measure FAS promoter activity. FAS promoter activity after expression of 1b core$^{ASC/VLV}$ mutant was not statistically different from 1b core^{WT} (Fig. 3.3d, 1b core^{WT} vs. core$^{ASC/VLV}$, p=0.5). In contrast, expression of 3a core$^{ASC/VLV}$ resulted in significantly higher FAS promoter activity than core^{WT} (Fig. 3.3e, 3a core^{WT} vs. core$^{ASC/VLV}$, p=0.000014). These results suggest that partial alteration in HCV-3a core protein processing had a measurable effect on FAS promoter up-regulation.

Since deletion of 18 amino acid residues from the C-terminus represents the fully processed form of HCV core protein (Liu et al., 1997; Yamanaka et al., 2002), we generated the truncated forms of the core proteins of both genotypes and studied their role in FAS up-regulation. Both 1b and 3a core proteins were truncated till amino acid 173 from the C-termini. Western blot analysis showed the presence of the core protein at a size consistent with the processed core protein (tCore^{173} in Fig. 3.3b). When the subcellular localization of the truncated core proteins was examined, a dramatic difference was observed (Fig. 3.3c). The 1b tCore^{173} protein localized primarily in the cytoplasm. A small number of cells (~15%) exhibited lipid droplet localization. In contrast, 3a tCore^{173} protein was primarily localized in the nucleus. No lipid droplet localization was observed.

When the truncated core proteins were used for measuring FAS promoter activity, expression of HCV-1b tCore^{173} was associated with significantly higher FAS promoter activity (Fig. 3.3d, 1b core^{WT} vs. 1b core^{173}, p=0.00009). In contrast, 3a tCore^{173} was no

longer able to up-regulate FAS promoter (Fig. 3.3e, 3a tCore^{173} vs. control, p=0.5). These results suggested that fully processed and nuclear HCV-3a core protein did not up-regulate FAS promoter.

3.4.5 Role of lipid droplet localization in FAS up-regulation by HCV core protein

Previous studies have identified that ^{164}YATG^{167} sequence of 1b core protein is crucial for lipid droplet localization (Hope et al., 2000; Hope et al., 2002). HCV-3a core encodes a phenylalanine instead of a tyrosine at position 164 (Fig. 3.1a). To study the effect of ^{164}YATG^{167} or ^{164}FATG^{167} sequences in FAS activation, this sequence was deleted from 1b or 3a core and the expression of the mutant proteins was confirmed by Western blotting (Fig. 3.4a and 3.4b). Immunofluorescence analysis revealed a decrease in the overall amount of core protein localizing to lipid droplets (Fig. 3.4c). These results did not only confirm the role of the ^{164}YATG^{167} motif in lipid droplet localization for 1b core protein, but also demonstrated that the ^{164}FATG^{167} motif was involved in directing HCV-3a core protein onto lipid droplets. When these mutants were used to determine FAS promoter activity, deletion of the ^{164}YATG^{167} or ^{164}FATG^{167} sequences in 1b or 3a core entirely abolished FAS promoter activation (Fig. 3.4d and 3.4e; control vs. 1b core$^{\Delta YATG}$, p=0.45, control vs. 3a core$^{\Delta FATG}$, p=0.54).

A proline knot motif (composed of two proline residues within the sequence ^{138}PLVGAP^{143}) of the 1b core protein is also critical for lipid droplet localization (Hope et al., 2002). The proline knot motif is conserved in HCV-3a core protein (Fig. 3.1a). To characterize the role of the proline knot motif in FAS activation, the two proline residues were changed to alanines (Fig. 3.4a). Western blot analysis demonstrated the expression

of the mutant core$^{P138A/P143A}$ proteins (Fig. 3.4b). Subcellular localization of the proline knot mutants showed a significant decrease in lipid droplet localization for both genotypes (Fig. 3.4c). When examined for FAS promoter analysis, expression of 1b and 3a core$^{P138A/P143A}$ mutants resulted in significantly higher FAS promoter compared to core^{WT} (Fig. 3.4d, 1b core^{WT} vs. 1b core$^{P138A/P143A}$, p=0.0002; Fig. 3.4e, 3a core^{WT} vs. 3a core$^{P138A/P143A}$, p=0.00003). These results suggest that the two lipid droplet localization motifs in HCV core protein had different effects on FAS promoter up-regulation.

3.4.6 Involvement of phenylalanine^{164} of HCV-3a core protein in FAS up-regulation

To investigate the role of phenylalanine^{164} of 3a core in FAS up-regulation, we mutated the phenylalanine^{164} to a tyrosine, which is present in the 1b core (Fig. 3.5a). Western blot analysis showed similar processing and expression levels of 3a core^{WT} or core^{F164Y} proteins (Fig. 3.5b). Subcellular localization of 3a core^{F164Y} protein was also very similar to the wild-type with the majority of the core protein localizing on lipid droplets around the nucleus (Fig. 3.5c). The 3a core^{F164Y} mutant was then used to examine FAS promoter activity. Expression of 3a core^{F164Y} was associated with significantly higher FAS promoter activity than control (Fig. 3.5c, control vs. 3a core^{F164Y}, p=0.0043). However, FAS promoter activity was significantly reduced after 3a core^{F164Y} expression in comparison to 3a core^{WT} (Fig.5c, 3a core^{WT} vs. core^{F164Y}, p=0.00057). These results indicate that phenylalanine^{164} of 3a core plays a major role in FAS promoter activation.

3.5 Discussion

Clinical investigations suggest that HCV-3a is a stronger steatogenic factor than HCV of other genotypes. However, the molecular mechanisms underlying this process have not been well characterized. In this study, we demonstrated that expression of HCV-3a core protein resulted in significantly stronger up-regulation of FAS promoter than 1b core. In addition, we identified that phenylalanine^{164} encoded by HCV-3a core protein was primarily responsible for this increased effect.

It has been well documented that regulated proteolytic cleavage of HCV core protein results in the presence of processed and unprocessed core proteins (Kato et al., 2003; Liu et al., 1997; Yamanaka et al., 2002). The regulated core protein processing plays a role in determining its function (Yamanaka et al., 2002; Moorman et al., 2003; Bergqvist et al., 2001). In this paper, we showed that partial prevention of processing by amino acid mutations at the carboxyl-terminus abolished FAS activation by HCV-1b core, but enhanced 3a core associated FAS activation (Fig. 3.3d and 3.3e). In contrast, only fully-processed HCV-1b core, but not 3a core, could up-regulate FAS promoter (Fig. 3.3d and 3.3e). These results indicated that regulated processing of core proteins of different HCV genotypes was differentially involved in FAS promoter regulation. The underlying mechanism is a subject for subsequent studies.

Since FAS is directly linked to intracellular lipid synthesis (Semenkovich et al., 1997; Sul et al., 1998) and lipid droplet is the major organelle for intracellular lipid storage (Martin et al., 2006), we investigated the role of lipid droplet localization of HCV core protein in FAS up-regulation. Consistent with previous studies (Hope et al., 2000; Hope et al., 2002), mutations of the proline knot and the Y/FATG sequences of HCV

core protein resulted in reduced lipid droplet localization. Somewhat to our surprise, the proline knot and Y/FATG mutations had different effects on FAS promoter activity (Fig. 3.4). While the expression of proline knot mutant of either 1b or 3a core proteins resulted in enhanced FAS promoter activity, YATG (1b) or FATG (3a) deletion totally abolished FAS promoter up-regulation. These results indicated that lipid droplet localization of HCV core protein did not play a major role in up-regulating FAS. Instead, the primary amino acid sequences, i.e., YATG (1b) and FATG (3a), were critical for FAS up-regulation. In addition, this short amino acid stretch might also be responsible for the stronger FAS activation by 3a core than 1b core. Indeed, changing the phenylalanine to tyrosine in HCV-3a core protein significantly reduced the degree of FAS promoter activation. This result suggested that the phenylalanine^{164} of 3a core was involved in FAS up-regulation.

The molecular mechanism for the involvement of phenylalanine^{164} of 3a core protein in FAS activation is not known. One possibility is that the phosphorylation status at this particular position of HCV core protein is important. Although serine phosphorylation of HCV-1 core protein has been demonstrated (Lu et al., 2002; Shih et al., 1995), whether the tyrosine^{164} of HCV-1b core protein is phosphorylated is yet to be determined. Alternatively, since HCV core interacts with numerous cellular proteins (Kang et al., 2005), the presence of a phenylalanine or a tyrosine at this position may influence these interactions, which in turn influence FAS activation. Future research will clarify this issue.

In conclusion, our research showed a stronger activation of FAS promoter by HCV-3a core than 1b core. This result increased our understanding of the mechanisms

by which HCV-3a infections are associated with higher prevalence and severity of steatosis.

Figure 3.1. Expression and subcellular localization of HCV-1b and -3a core proteins. A) Amino acid sequence comparison of HCV-1b and -3a core proteins. **B)** Expression of HCV core proteins. Huh-7 cells were transfected with pEF/cyto/myc vector (control), or plasmids expressing HCV-1b core or -3a core. Core proteins were analyzed by Western blotting using a core-specific antibody 48 hr after transfection. The levels of β-actin were blotted for protein loading control. **C) and D)** Subcellular localization of HCV core proteins in Huh-7 cells. After transfection, HCV core protein was detected with a core-specific monoclonal antibody and a secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes). Lipid droplets and endoplasmic reticulum (ER) were detected by Oil Red O (**C**) or a calnexin-specific antibody (**D**), respectively. The nuclei were detected by DAPI. Scale bar, 20 μm.

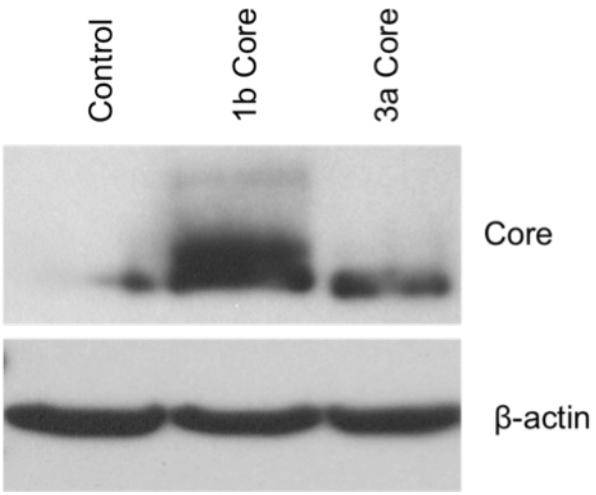
A

```
1b Core    1MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRAPRKTSERSQPRGRRQPI
3a Core    1MSTLPKPQRKTKR I TNRRPQDVKFPGGGQIVGGVYVLPRRGPRLGLRATRKTSERSQPRGRRQPI

1b Core   66PKARRPEGRTWAQPGYPWPLYGNEGLGWAGWLLSPRGSRPSWGPTDPRRRSRNLGKVIDTLTCG
3a Core   66PKARRSEGRSWAQPGYPWPLYGNEGCGWAGWLLSPRGSRPSWGPNDPRRRSRNLGKVIDTLTCG

1b Core  130FADLMGYIPLVGAPLGGAARALAHGVRVLEDGVNYATGNLPGCSFSIFLLALLSCLTTPASA
3a Core  130FADLMGYIPLVGAPVGGVARALAHGVRALEDGI NFATGNLPGCSFSIFLLALFSCL IHPAAS
```

B



C

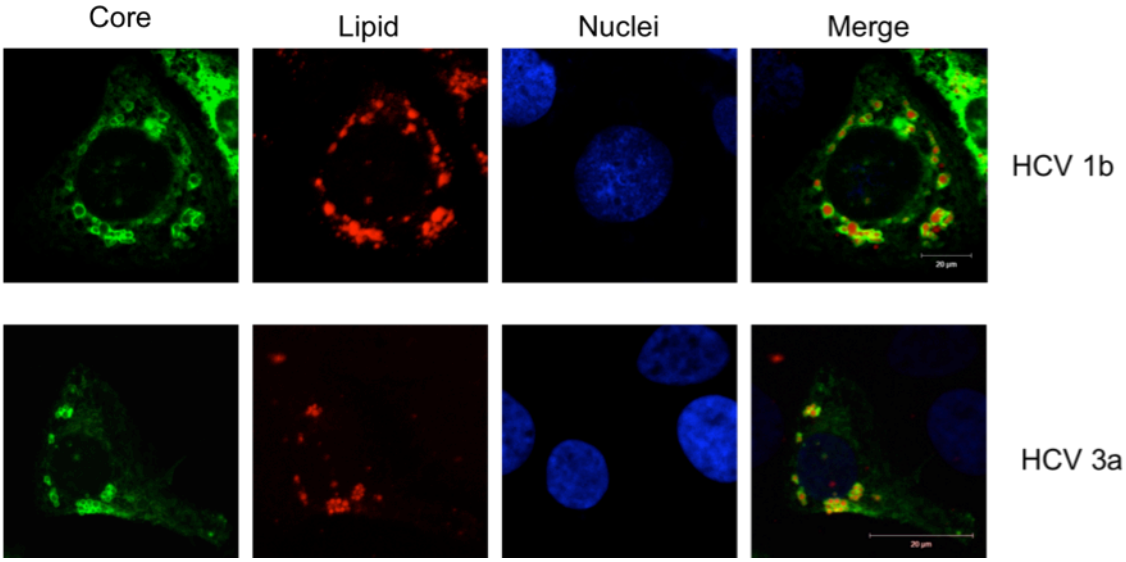


Figure 3.1

D

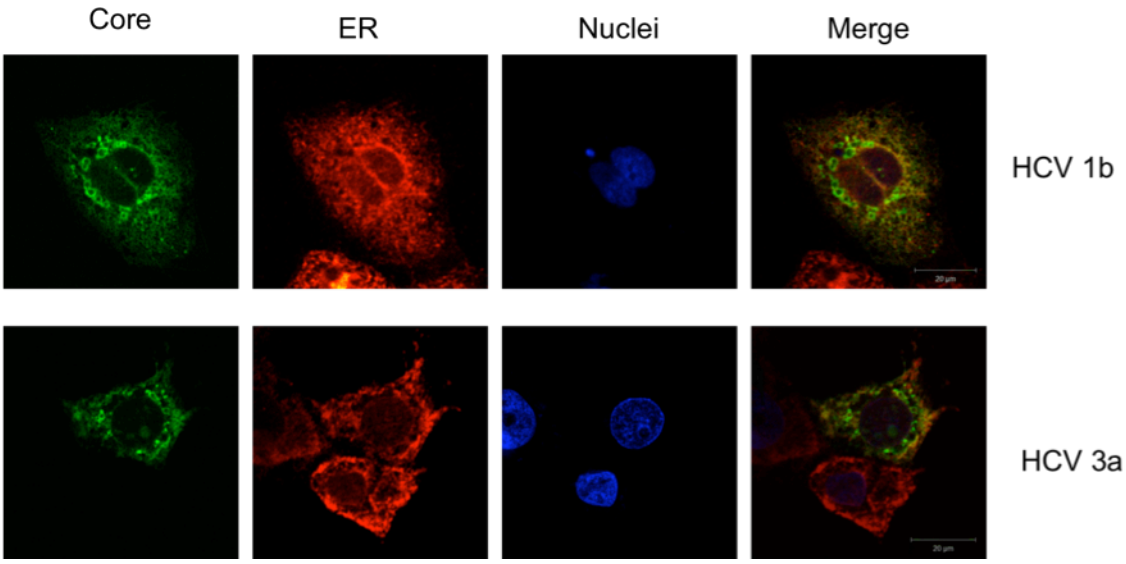
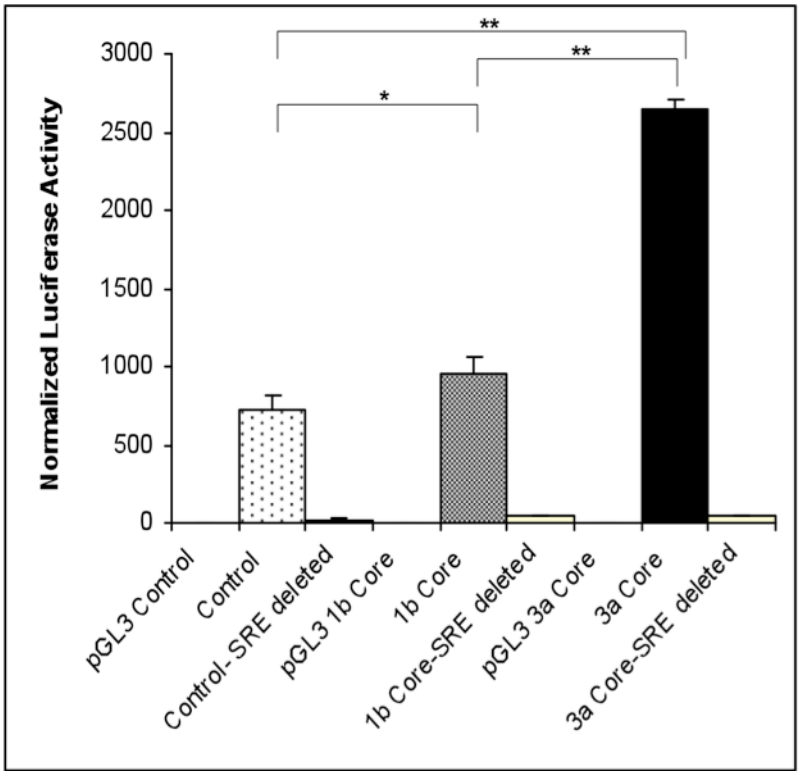


Figure 3.1

Figure 3.2. HCV core protein up-regulates FAS promoter in Huh-7 cells in an SREBP-1-dependant manner. A) Huh-7 cells were co-transfected with HCV-1b or -3a core-expressing plasmids, together with pGL3-basic vector, a FAS promoter-luciferase reporter plasmid, or an SRE-deleted FAS promoter reporter. Plasmid pRL-SV40 encoding Renilla luciferase was also included in the co-transfection experiment. Luciferase assay was carried out 48 hr after transfection. FAS promoter activity was expressed as relative light units (RLU) after normalization of the firefly luciferase against the Renilla luciferase levels followed by normalization against the core protein levels in Fig. 1b. The statistical difference between the samples was demonstrated as * if $p \leq 0.05$ or ** if $p \leq 0.01$. **B)** Huh-7 cells were co-transfected with HCV-1b or -3a core-expressing plasmids, a FAS promoter-luciferase reporter plasmid, together with pcDNA3.1(+) vector or a plasmid expressing a dominant-negative mutant of SREBP-1. Luciferase assay was performed 48 hr after transfection to determine FAS promoter activity.

A


Normalized Luciferase Activity
3000
2500
2000
1500
1000
500
0
**
**
*
pGL3 Control
Control
Control- SRE deleted
pGL3 1b Core
1b Core
1b Core-SRE deleted
pGL3 3a Core
3a Core
3a Core-SRE deleted

B

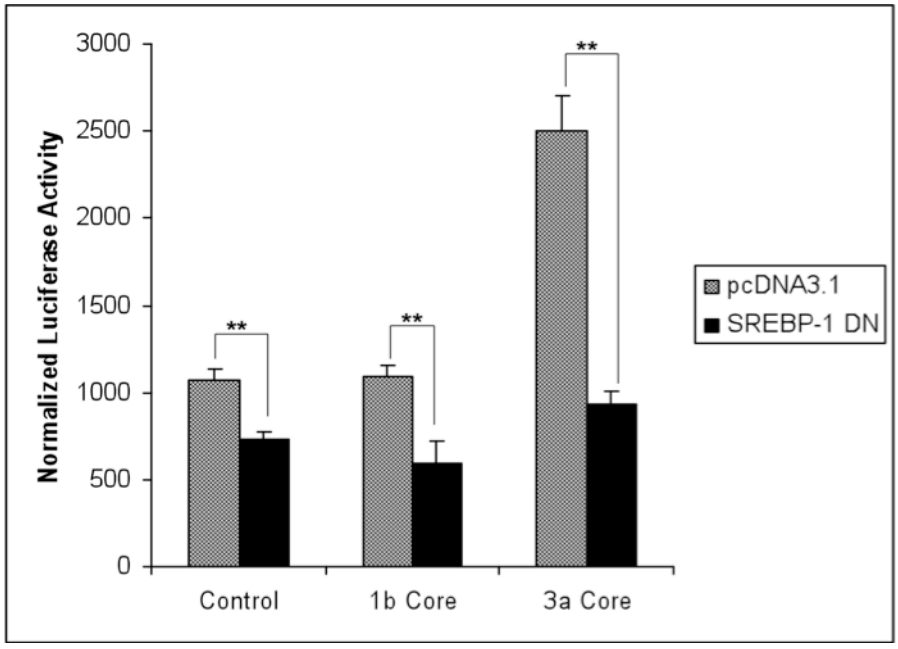

Normalized Luciferase Activity
3000
2500
2000
1500
1000
500
0
**
**
**
pcDNA3.1
SREBP-1 DN
Control
1b Core
3a Core

Figure 3.2

Figure 3.3. The role of HCV core proteolytic processing on FAS promoter up-regulation. A) HCV-1b or -3a core processing mutants were generated by amino acid substitutions at three positions at the carboxyl-terminus (A180V/S183L/C184V). **B)** Expression of HCV core processing mutant proteins. Huh-7 cells were transfected with pEF/cyto/myc vector (control), or plasmids expressing 1b core$^{ASC/VLV}$ or 3a core$^{ASC/VLV}$. Truncations were also created by deleting the C-terminal 18 amino acids of 1b or 3a core proteins (core^{173}) and used in transfection. The expression of mutant core proteins was analyzed by Western blotting using a core-specific antibody 48 hr after transfection. **C)** Subcellular localization of the processing mutant core proteins in Huh-7 cells. HCV core proteins were stained with a core-specific monoclonal antibody. Lipid droplets were stained by Oil Red O and the nuclei were stained by DAPI. Scale bar, 20 μm. **D)** and **E)** FAS promoter activity after expression of HCV-1b (Fig.3d) or -3a (Fig.3e) core processing mutants. Huh-7 cells were co-transfected by a FAS promoter-luciferase reporter and pEF/cyto/myc vector (control), or plasmid expressing core^{WT}, or core$^{ASC/VLV}$, or core^{173}. Luciferase assay was performed 48 hr after transfection to determine FAS promoter activity.

A


1b core^WT
175SIFLLALLSCLTTPASA191
1b core^ASC180/3/4VLV
175. . . . .V . . VL. 191
3a core^WT
175SIFLLALFSCLIHPAAS191
3a core^ASC180/3/4VLV
175. . . .. V. . VL. 191

B

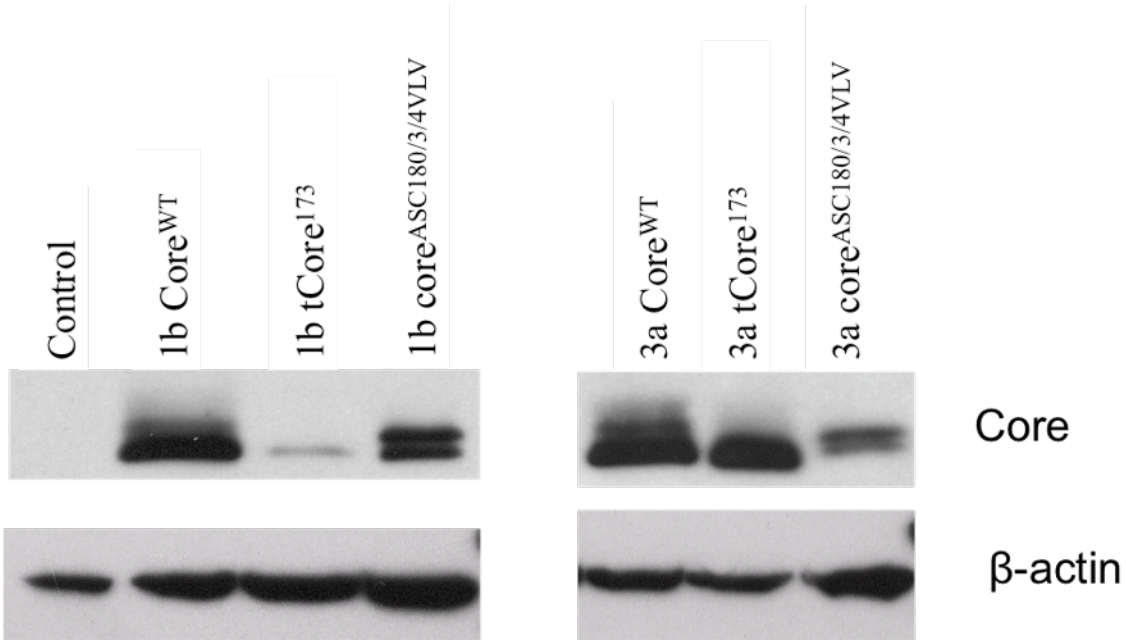

Control
1b Core^WT
1b tCore^173
1b core^ASC180/3/4VLV
3a Core^WT
3a tCore^173
3a core^ASC180/3/4VLV
Core
β-actin

Figure 3.3

C

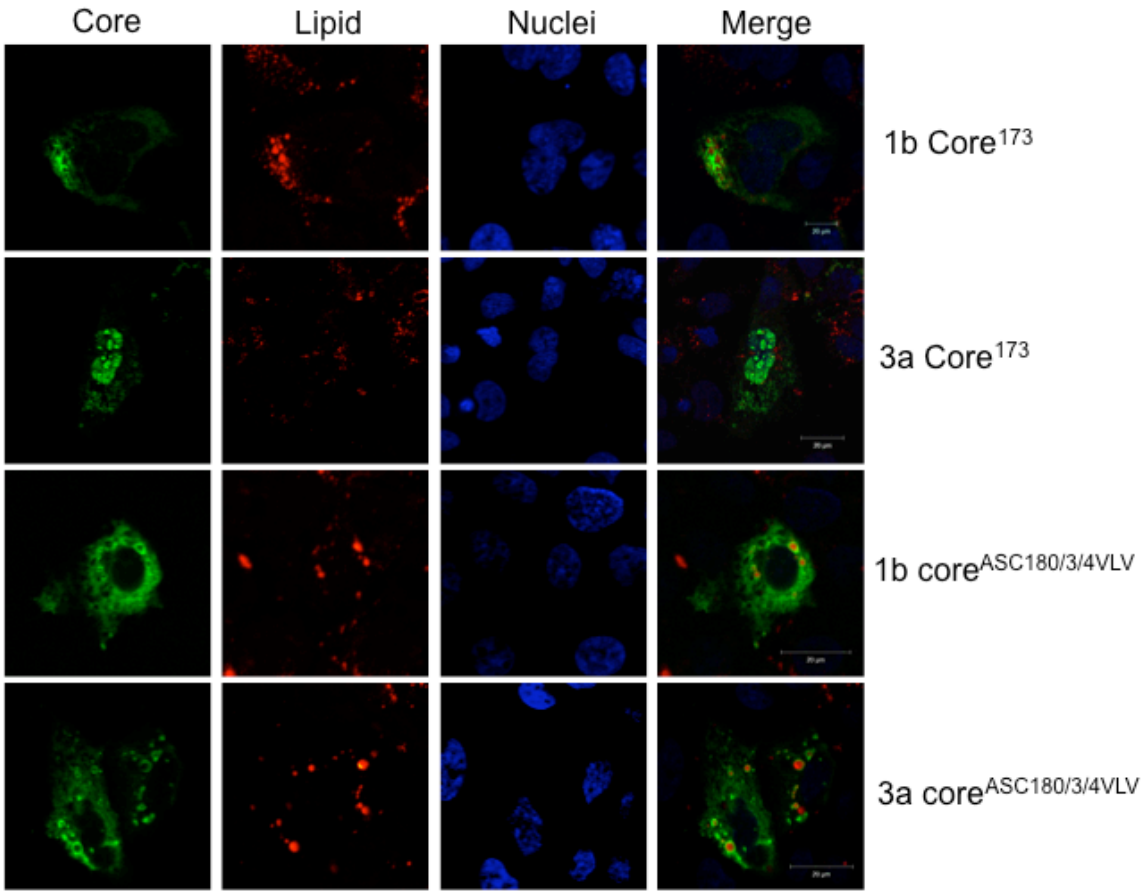
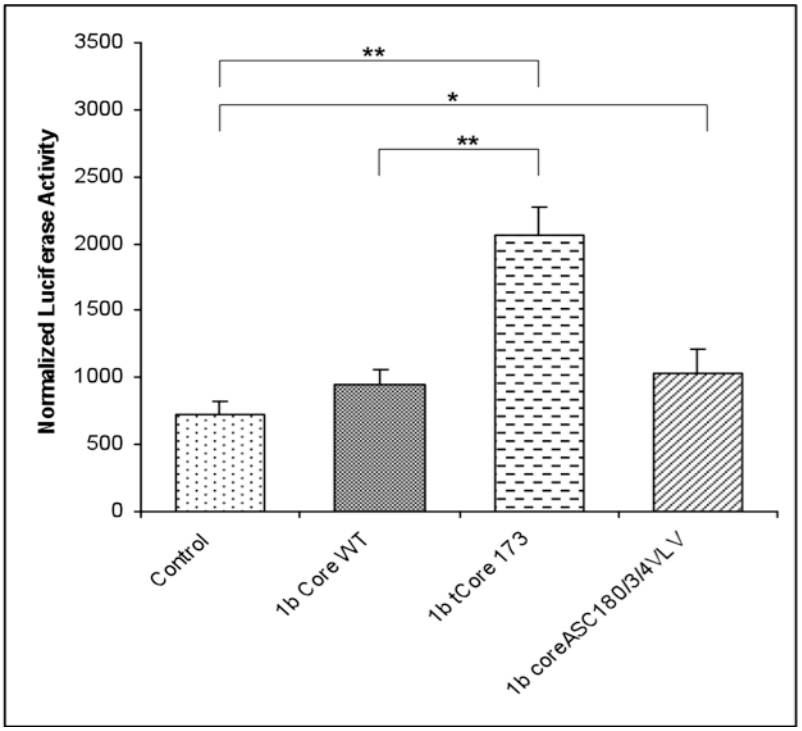

Core
Lipid
Nuclei
Merge
1b Core173
3a Core173
1b coreASC180/3/4VLV
3a coreASC180/3/4VLV

Figure 3.3

D


Normalized Luciferase Activity
3500
3000
2500
2000
1500
1000
500
0
**
*
**
Control
1b Core WT
1b tCore 173
1b coreASC180I3I4VLV

E

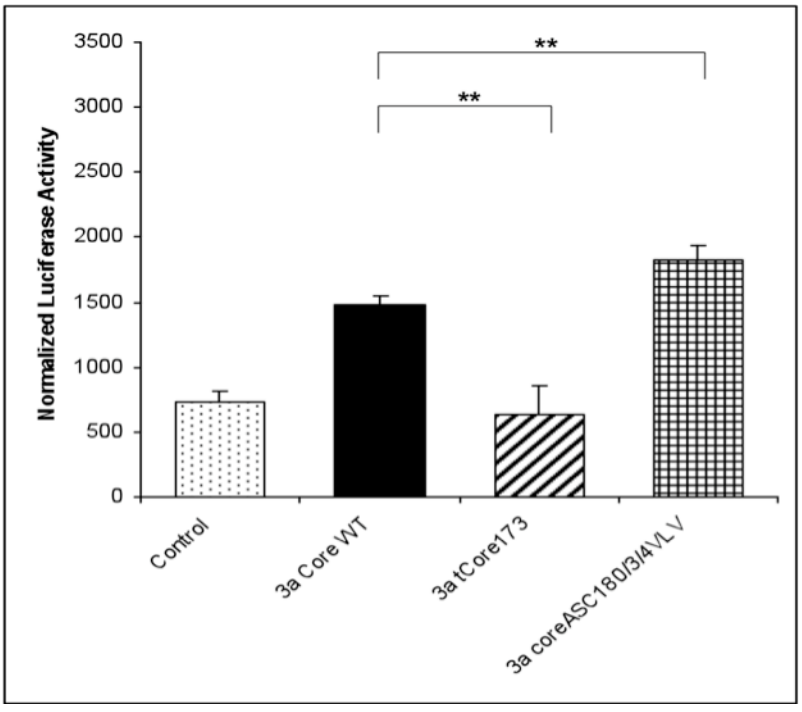
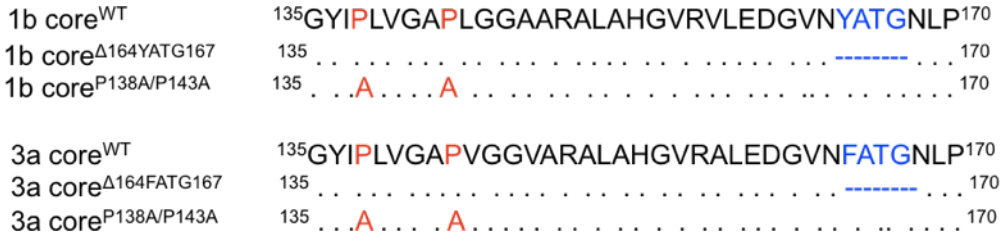

Normalized Luciferase Activity
3500
3000
2500
2000
1500
1000
500
0
**
**
Control
3a Core WT
3a tCore173
3a coreASC180I3I4VLV

Figure 3.3

Figure 3.4. Effect of lipid droplet localization of HCV core proteins on FAS promoter up-regulation. A) Lipid droplet localization motifs in HCV core protein. Two prolines at positions 138 and 143 (red) representing a proline knot were mutated to alaines. The ^{164}YATG^{167} motif of 1b core or the ^{164}FATG^{167} sequence of 3a core was deleted. **B)** Expression of HCV core lipid droplet localization mutants determined by Western blotting using a core-specific antibody after transfection in Huh-7 cells. **C)** Subcellular localization of core proteins with lipid droplet localization motif mutations. HCV core proteins were stained with a core-specific monoclonal antibody. Lipid droplets were stained by Oil Red O and the nuclei were stained by DAPI. Scale bar, 20 μm. **D)** and **E)** FAS promoter activity after expression of HCV core lipid droplet localization mutants in 1b core (3d) or 3a core (3e). Huh-7 cells were co-transfected with a FAS promoter-luciferase reporter, together with pEF/cyto/myc vector (control), or plasmid expressing core^{WT}, or core$^{\Delta Y/FATG}$, or core$^{P138A/P143A}$. Luciferase assay was performed 48 hr after transfection.

A


1b core^WT
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1b core^Δ164YATG167
135 . . . . . -------- . . . 170
1b core^P138A/P143A
135 . . .A . . . .A 170
3a core^WT
135GYIPLVGAPVGGVARALAHGVRALEDGVNFATGNLP170
3a core^Δ164FATG167
135 . . . . . -------- . . . 170
3a core^P138A/P143A
135 . . .A . . . .A 170

B

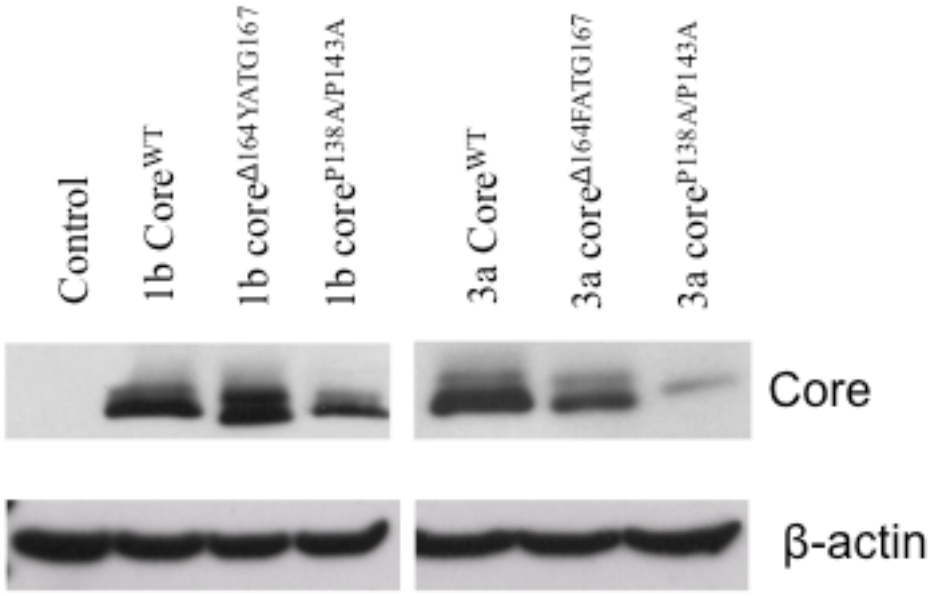
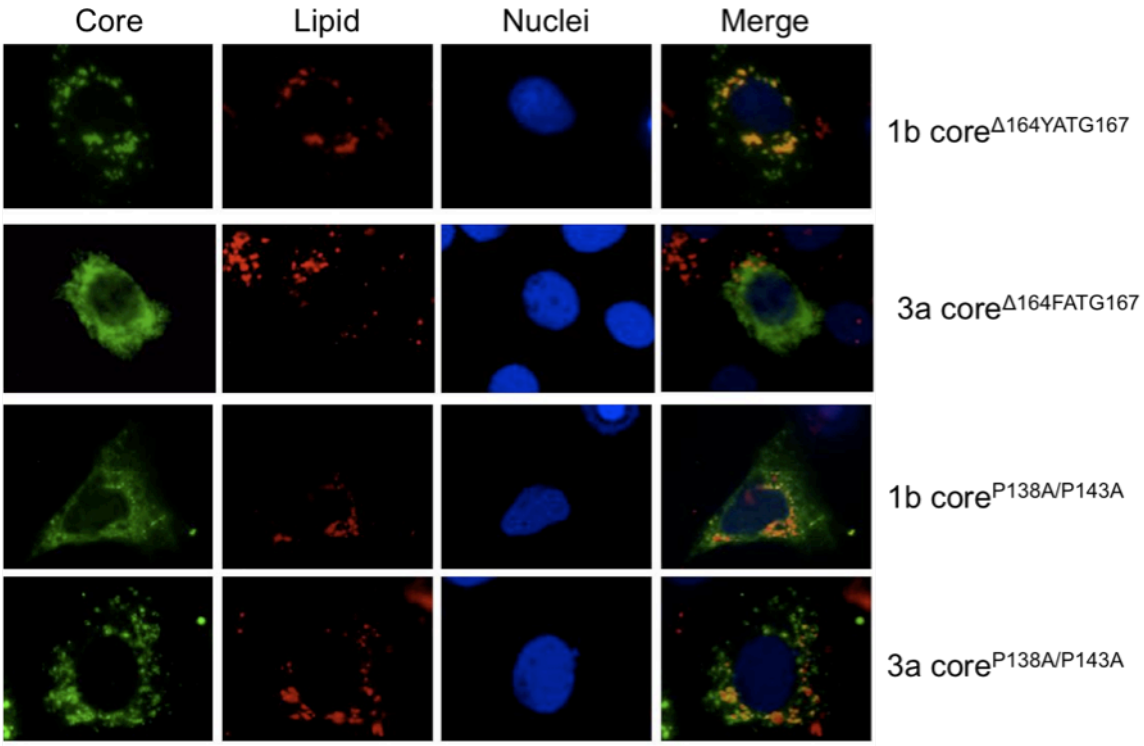

Control
1b Core^WT
1b core^Δ164YATG167
1b core^P138A/P143A
3a Core^WT
3a core^Δ164FATG167
3a core^P138A/P143A
Core
β-actin

Figure 3.4

C


Core
Lipid
Nuclei
Merge
1b core$^{\Delta 164YATG167}$
3a core$^{\Delta 164FATG167}$
1b core$^{P138A/P143A}$
3a core$^{P138A/P143A}$

D

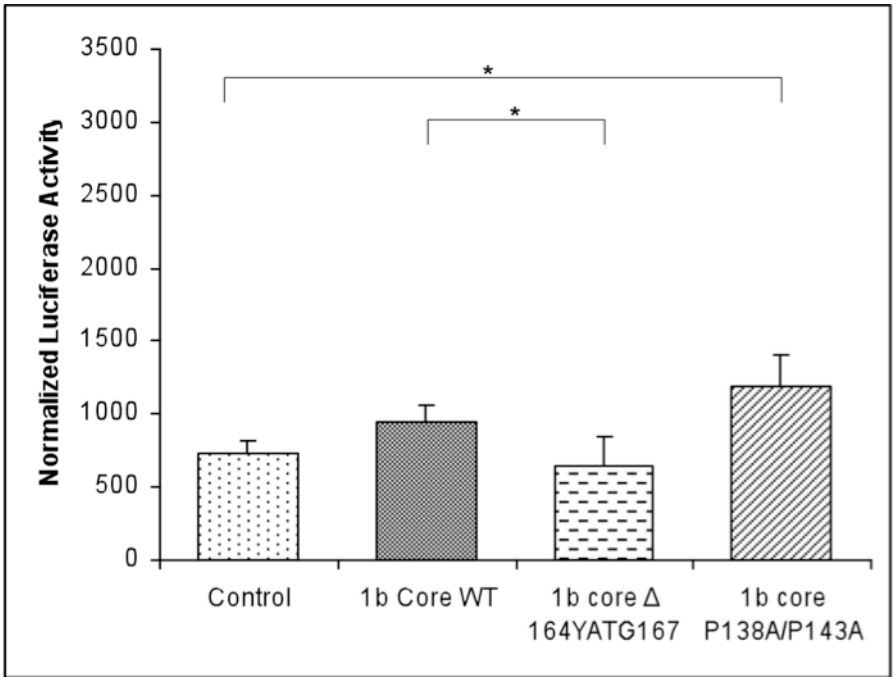

Normalized Luciferase Activity
3500
3000
2500
2000
1500
1000
500
0
*
*
Control
1b Core WT
1b core Δ 164YATG167
1b core P138A/P143A

Figure 3.4

E

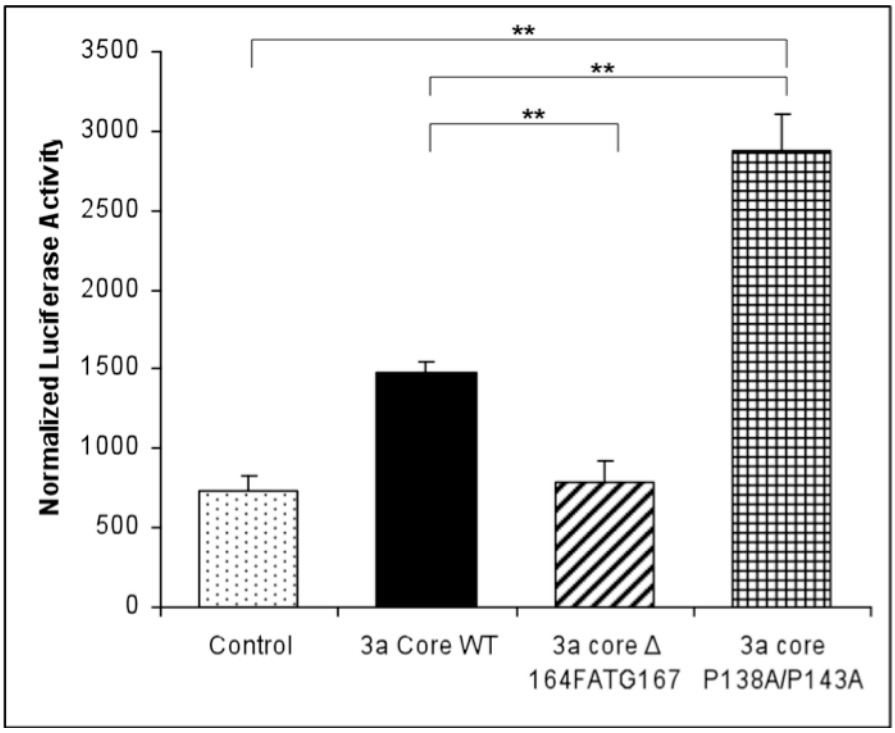
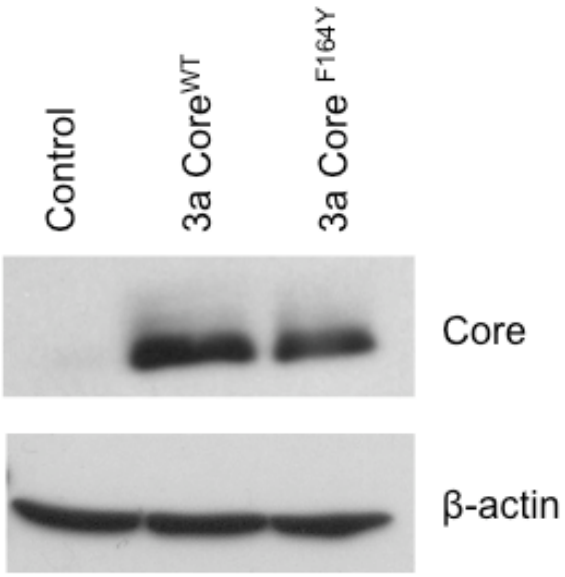

**
**
**
3500
3000
2500
2000
1500
1000
500
0
Normalized Luciferase Activity
Control
3a Core WT
3a core Δ 164FATG167
3a core P138A/P143A

Figure 3.4

Figure 3.5. Involvement of F^{164} in HCV- 3a core protein in FAS promoter up-regulation. A) Expression of 3a $core^{F164Y}$. Huh-7 cells were transfected with pEF/cyto/myc vector (control), or plasmid expressing 3a wild-type or F164Y mutant core proteins. Western blot analysis was performed 48 hr after transfection using a core-specific antibody. **B)** Subcellular localization of the 3a $core^{F164Y}$ mutant. After transfection, the core protein was stained with a core-specific monoclonal antibody and a secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes). Lipid droplets were stained by Oil Red O and the nuclei were stained by DAPI. Scale bar, 20 μm. **C)** FAS promoter activity after expression of 3a $core^{F164Y}$ mutant. Huh-7 cells were co-transfected with a FAS promoter-luciferase reporter, together with pEF/cyto/myc vector (control), or plasmid expressing 3a $core^{WT}$, or $core^{F164Y}$ mutant. Luciferase assay was performed 48 hr after transfection.

A


Control
3a Core^WT
3a Core F164Y
Core
β-actin

B

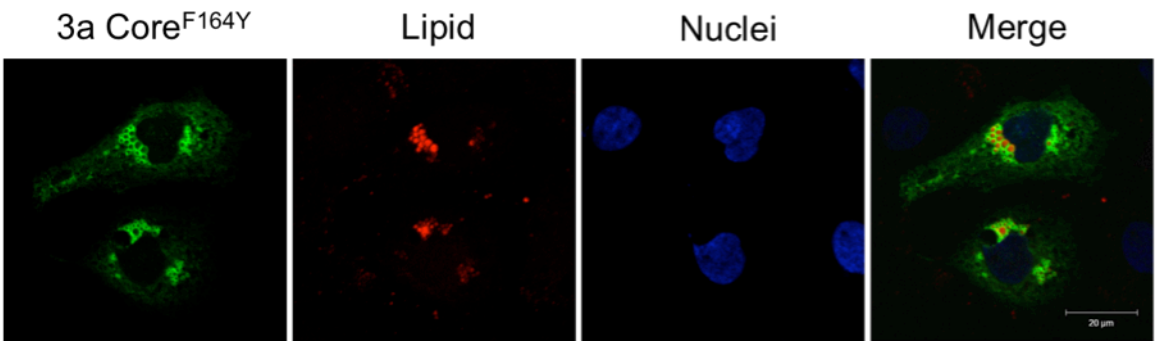

3a Core^F164Y
Lipid
Nuclei
Merge

Figure 3.5

C

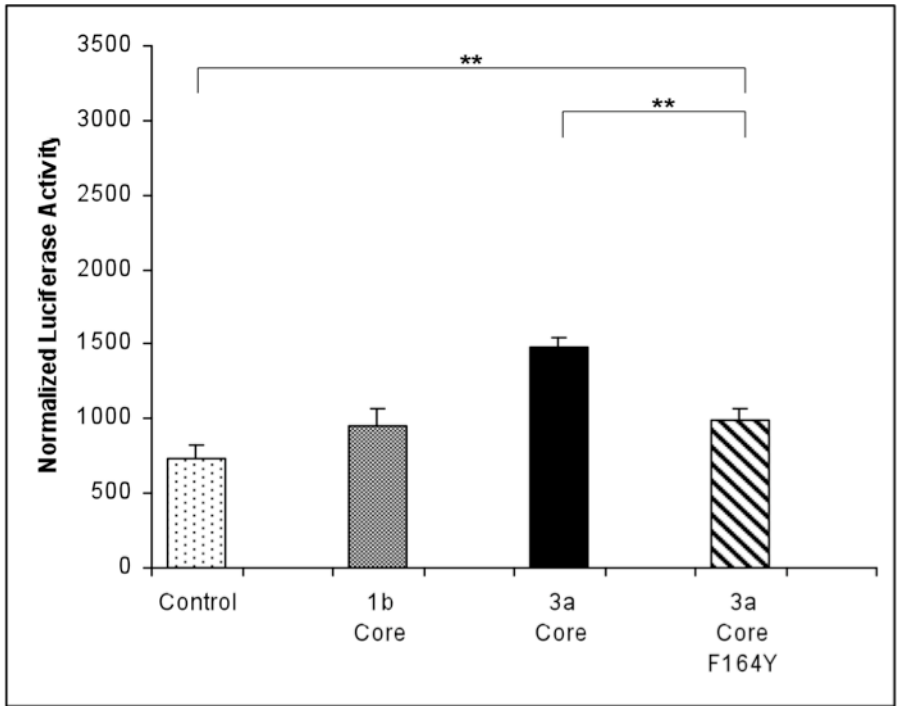

Normalized Luciferase Activity
3500
3000
2500
2000
1500
1000
500
0
**
**
Control
1b Core
3a Core
3a Core F164Y

Figure 3.5

4.0 HEPATITIS C VIRUS GENOTYPE-3A CORE PROTEIN ENHANCES STEROL RESPONSE ELEMENT BINDING PROTEIN-1 TRANSCRIPTION AND CLEAVAGE VIA INCREASED PROTEIN KINASE B/AKT ACTIVITY

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4.1 Abstract

Hepatitis C virus genotype-3a (HCV-3a) is directly linked to the development of steatosis, or fatty liver. Previously, we studied the effects of HCV-3a core protein on the promoter activity of fatty acid synthase (FAS), a major enzyme involved in *de novo* lipid synthesis that is regulated by Sterol Response Element Binding Protein-1 (SREBP-1). The signal transduction pathways responsible for FAS up-regulation by HCV-3a core protein are not understood. In this study we further investigated the role of the HCV-3a core protein in altering SREBP-1 activity by examining its effect on Akt, an important cell signaling protein.

Genomic replicon expressing cells were established that contain the genotype 3a core in a genotype 1b N strain backbone. The chimeric genotype 3a replicon up-regulated transcriptional activity of SREBP-1c in an SRE and LXRE dependent manner in comparison to the genotype 1b replicon. SREBP-1 processing into the mature form was also enhanced in genotype 3a core expressing cells. Further examination revealed that Akt-1 and Akt-2 phosphorylation was increased in the presence of genotype 1b and 3a core proteins, with a greater effect of 3a core. When Akt activity was inhibited using chemical inhibitors LY294002 or AktVIII or by using dominant negative Akt plasmids, the effect of 3a core protein on transcriptional activity from the SRE and SREBP-1c promoter activity was inhibited. Knocked down Akt-1 or Akt-2 with shRNAs had a similar effect. These results indicated a role for HCV-3a core protein in regulating Akt activity, especially those involved in regulation of lipogenic gene expression.

4.2 Introduction

Infection with hepatitis C virus (HCV) results in the development of hepatitis, steatosis, fibrosis, cirrhosis, hepatocellular carcinoma and the eventual need for liver transplantation. Different genotypes of the virus, based on sequence homology, cause different clinical pathologies and also differ in their response rates to interferon/ Ribavirin combination therapy. Clinical evidence has shown that genotype 3a may have a direct cytopathic effect on hepatocytes, with approximately 70% of patients developing steatosis, or fatty liver, in the absence of other contributing factors such as obesity, diabetes and alcoholism. Furthermore, clearance of the genotype 3a virus decreases or abolishes the presence of steatosis (Castera et al., 2004). Other genotypes do not appear to contribute as directly to the development of steatosis. The presence of steatosis can hamper successful clearance of the virus during therapy as well as increase the progression of the liver to cirrhosis.

Genotype 1b core protein can enhance the processing of Sterol Response Element Binding Protein-1 (SREBP-1) and SREBP-2, key transcription factors that activate genes involved in lipid and cholesterol metabolism such as Fatty Acid Synthase (FAS) through binding of the Sterol Response Element in the gene promoters (Horton et al., 2002; Latasa et al, 2000). SREBP-1 and SREBP-2 are localized on the endoplasmic reticulum in a complex with SREBP Cleavage Activating Protein (SCAP) until low cholesterol and/or lipid levels or increased insulin induces the complex to move into the Golgi, where SREBP-1/2 is cleaved and the amino terminal end is released into the cytoplasm to localize in the nucleus and regulate transcription of lipid metabolism genes (Bengoechea-Alonso and Ericsson, 2007; Raghow et al., 2008). The influence of HCV core on these

transcription factors could play an important role in the development of steatosis during chronic HCV infection.

Previous research has shown that the core protein of genotype 3a HCV may have a differential mechanism in regulating lipid metabolism during infection in comparison to genotype 1b, although the exact molecular mechanisms are not well understood (Abid et al., 2005; Hourioux et al., 2007; Pazienza et al., 2008; Piodi et al., 2008; Waris et al., 2007). The core protein localizes to lipid droplets in transfected and infected Huh7 cells and abrogation of this localization decreases the production of infectious virus particles, indicating that localization of core on lipid droplets is important for virus assembly (Miyanari et al., 2007). Genotype 1b HCV core has been shown to influence Microsomal Transfer Protein (MTP) activity, which is the enzyme involved in packaging triglycerides into Very Low Density Lipoproteins (VLDL) for secretion, and subsequently decreases apolipoprotein B-100 secretion (Perlemuter et al., 2002). Transgenic mice expressing core protein also develop steatosis (Moriya et al., 1998). Furthermore, a recent study has shown that FAS protein expression is substantially increased in JFH-1 cell culture infection; when FAS expression was inhibited, viral production and entry were decreased (Yang et al., 2008). These data suggest that the influence of HCV on lipid metabolism may not only be important for the development of steatosis, but also for viral infection and therefore could be a viable drug target.

Our previous study has shown that genotype 3a core protein is able to up-regulate transcription of FAS to a greater extent than genotype 1b core protein in an SREBP-1 dependant manner (Chapter 3: Jackel-Cram et al., 2007). In this study we have further investigated how genotype-3a and 1b core protein influence SREBP-1 transcription and

processing through increasing activity of Akt-1 and Akt-2. These results provide further information on the important differences between genotype 1b and 3a HCV infection and how it may lead to the development of steatosis.

4.3 Materials and Methods

4.3.1 Plasmids, Antibodies and Reagents.

A plasmid containing the SREBP-1c promoter region (-571/+90) was provided by Dr. Lefai (Dif et al., 2006). The promoter truncations and SRE and LXRE deletions were generated using site directed mutagenesis as previously described (Oem et al., 2008). A plasmid containing His_6-tagged Akt-2 was generated for use in His-tagged protein pull down for Akt-2 phosphorylation studies. HCV core antibody was purchased from Anogen and NS5b antibody was obtained from Dr. Joyce Wilson. SREBP-1 antibody was obtained from Santa Cruz Biotechnology. Antibodies for total Akt-1, total Akt-2, phospho-Ser Akt, and β-actin were purchased from Cell Signaling Technology. LY294002 was purchased from EMD Biosciences.

4.3.2 Transfections.

Huh7 cells were transfected using calcium phosphate precipitation as previously described (Jackel-Cram et al., 2007).

4.3.3 Generation of 1b/3a chimeric replicon.

Huh7 cells stably expressing an HCV-1b N genomic replicon were kindly provided by Dr. S. Lemon. Genotype 3a core coding sequence was cloned in place of the 1b core protein in the N replicon through PCR-mediated recombination. RNA generated by *in vitro* transcription from linearized plasmid DNA was electroporated into replicon-cured Huh7 cells (provided by Dr. Joyce Wilson). Cells were selected using G418 and the resultant colonies were examined for replicon expression using Western blots and RT-PCR. For transient replication assays, plasmids containing a T7 promoter and the HCV genomic (HCV-1b N or HCV-3a core/1b N) or subgenomic (HCV-1b N or HCV-1b N GND mutant) virus sequences with a luciferase reporter gene were used to generate RNA with an Ambion T7 megascript kit. 2.5 μg of RNA was transfected into Huh7 cells using DMRIE-C (Invitrogen). Cells were lysed in Passive Lysis Buffer (Promega) and assayed for luciferase activity at selected time points.

4.3.4 Western blot analysis and Histidine-tagged protein pull-down assay.

All cells were cultured with Dulbecco's Modified Eagles Medium containing 1% Fetal Bovine Serum for 24 hours prior to harvesting. Cells were harvested 48 hours after transfection with Cell Lysis Buffer (Cell Signaling Technology) containing 10 μM phenylmethylsulphonyl fluoride (PMSF) according to the manufacturer's protocol. Total protein was quantified. For His-tagged protein pull-down, equivalent amounts of cell lysates were added to nickel resin (Qiagen) overnight at 4 °C. The resin was washed 4X in Phosphate Buffered Saline (pH 7.2) and then eluted in elution buffer. For Western blotting, total protein or His-tagged pulled down proteins were subjected to SDS-PAGE,

blotted onto nitrocellulose, blocked in 3% Bovine Serum Albumin, and placed in primary antibody overnight. Blots were washed and incubated in secondary antibody (Li-Cor) for one hour and then washed again. Blots were then scanned using a Li-Cor Odyssey scanner at 600 nm (anti-mouse secondary) or 700 nm (anti-rabbit secondary) and fluorescent intensity of the resulting bands was quantified using the Odyssey software.

4.3.5 Luciferase assays.

Cells were plated at 4 x 10^4 per well of 24-well plates and cultured overnight. Cells were transfected with a maximum of 1.05 μg of plasmid including a Renilla Luciferase internal control. Media was changed 24 hours after transfection to DMEM with 1% FBS and appropriate inhibitors were added if necessary. Cells were washed and harvested in Passive Cell Lysis Buffer and luciferase activity was assayed according to the protocol of the Promega Dual Luciferase Assay system. Luciferase levels were normalized to the level of Renilla luciferase activity and statistical significance was determined by a student's *t* test.

4.3.6 Generation of Akt-1 and Akt-2 knock out cells.

Akt-1 or Akt-2-specific small hairpin (sh) RNA lentiviral constructs (Open Biosystems) were co-transfected with lentivirus packaging plasmids into HEK293T cells. The resulting lentiviral particles were used to infect Huh7 cells. 24 hours post-infection, 2.5 mg/ml of puromycin was added to select for infected cells. Akt-1 or Akt-2 knock-down was confirmed by Western blotting. A non-silencing construct was used to generate control cells (Fig 4.5).

4.4 Results

4.4.1 Generation and characterization of HCV chimeric replicon.

In order to compare the actions of genotype 1b core to genotype 3a core protein, we established a Huh7 cell line stably expressing a replicon containing 3a core protein in a full-length 1b genomic replicon backbone. Core protein levels were decreased in the genotype 3a chimeric replicon (Fig 4.1b) and in the transient transfections (Fig 4.1a). However, NS5b levels were only slightly lower in the 3a core/ 1b replicon. Also, the level of transient replication of the 1b replicon and 1b/3a core replicon was determined using a luciferase reporter-replicon construct that was transiently transfected into Huh7 cells and monitored for replication levels for 72 hours. Transient replication levels were similar for 1b and 1b/3a core replicons (Fig 4.1c).

4.4.2 HCV 3a core protein increases transcriptional activity from the SRE and LXRE transcription factor binding motifs.

Our previous findings indicated that 3a core can increase the transcription of FAS in an SREBP-1 dependent manner. SREBP-1c transcription is regulated by a positive feedback loop in which it contains an SRE responsive element along with a Liver X Receptor Responsive Element (LXRE). LXR is also an important transcription factor for genes involved in lipid and cholesterol synthesis (Dif et al., 2006). We first examined the effect of core protein on the SRE and LXRE binding elements alone using a luciferase assay. HCV-3a core significantly increased transcriptional activity from SRE in comparison to 1b core or control cells in both transient transfections and replicon

expressing cells. Transcriptional activity from LXRE was also increased by 3a core protein, but to a lesser extent than SRE indicating a much stronger effect of 3a core protein on SREBP-1 (Fig. 4.2a, b). Interestingly, 1b core appeared to have a stronger effect on LXRE than 3a core. Using deletions of the SRE and LXRE sequences (Fig. 4.2c, d), we showed that 3a core had also increased SREBP-1c transcriptional activity in comparison to 1b core and the control by luciferase reporter assays. This effect was evident using both transient core transfections (Fig. 4.2c) and the chimeric replicon expressing cells (Fig. 4.2d), with a slightly enhanced effect on SREBP-1c transcription in replicon expressing cells. Deletion of the SRE and LXRE motifs resulted in significantly decreased transcriptional activity from the SREBP-1c promoter in control, 1b core and 3a core transiently transfected and replicon cells. However, significantly increased SREBP-1c promoter activity was not observed in the presence of 1b core or the 1b replicon, despite the increased activity from SRE or LXRE alone, suggesting that 1b core may not influence the SREBP-1c promoter in the same manner or to the same extent as 3a core. This result further emphasizes the role of 3a core in increasing transcription of lipid metabolism genes through SREBP-1c and also suggests a role for the LXR in mediating the effects of the HCV core protein.

4.4.3 Processing of SREBP-1 is increased by the presence of HCV core protein.

In addition to transcriptional regulation, SREBP-1 is regulated post-translationally by cleavage into a mature, 68 kDa nuclear localized transcription factor. Therefore, we wanted to examine the cleavage of SREBP-1 in the presence of 1b or 3a core proteins. Our experiments demonstrated HCV-3a core increased cleavage of SREBP-1 into the

mature form in both transient transfections (Fig. 4.3a) and in the chimeric 1b/3a replicon cells (Fig. 4.3b) than HCV-1b core. Increased processing and/or stabilization of the mature SREBP-1 may contribute to the increased transcriptional activity from the SRE that we have observed.

4.4.4 Akt-1 and Akt-2 phosphorylation is increased in the presence of HCV core protein.

As previous studies had shown that Akt activity is involved in modulating SREBP-1 activity (Porstmann et al., 2005), we wanted to examine the level of Akt phosphorylation in the presence of 1b or 3a core protein. Three isoforms of Akt exist that have different effects on cell signaling. Akt-1 is primarily involved in cell survival while Akt-2 is potentially involved in insulin resistance (Cho et al., 2001). Our results demonstrated an increased phosphorylation of the Ser-473 residue of Akt-1 in genotype 3a core expressing cells in comparison to genotype 1b core expressing cells and both core proteins induced phosphorylation to a greater extent than the control cells (Fig. 4.4a, b). Similarly, a small increase in Ser-474 phosphorylation of Akt-2 was observed in the presence of genotype 3a and 1b core. (Fig. 4.4c, d). Densitometry analysis from three experiments to calculate a ratio between total Akt-2 and phosphorylated Akt-2 showed increased Akt-2 phosphorylation in the presence of core protein. The increased phosphorylation of Akt-1 and Akt-2 by HCV core could be abrogated by a PI3K inhibitor LY294002, indicating that activation of both Akt-1 and Akt-2 by HCV core is through PI3K (Fig. 4.4). These results suggest a possible role of Akt-2 in modulation lipid metabolism by HCV core.

4.4.5 Inhibition of Akt activity causes decreased SREBP-1 activation by HCV core protein.

Activity of Akt has been linked to the processing of SREBP-1c and inhibition of Akt also decreases SREBP-1 activity (Porstmann et al., 2005). As our previous experiments showed an increased amount of mature SREBP-1c in cells expressing HCV-3a core, we wanted to examine whether inhibition of Akt also affected SREBP-1 activity in the presence of core protein. Firstly, we examined the effect of Akt inhibition on the SREBP-1c promoter in the presence of HCV core proteins. For this purpose, we inhibited Akt activity using the PI3K inhibitor LY294002 or an Akt isoform specific inhibitor AktVIII. Inhibition of PI3K by LY294002 and Akt-1 and/or Akt-2 by AktVIII in transiently transfected and replicon cells significantly inhibited SREBP-1c promoter activation by HCV core (Fig. 4.5). These results suggest both Akt-1 and Akt-2 are involved in SREBP-1c promoter activation by HCV core.

To investigate whether Akt is also involved in activating SREBP-1 processing by HCV core, we again used LY294002. As shown in Fig. 4.3, treatment with LY294002 drastically decreased mature SREBP-1 level in comparison to control-treated cells. Consistently, when we assayed the SRE-driven luciferase activity as a read-out of SREBP-1 activity, the PI3K inhibitor LY294002 significantly decreased SRE-driven transcriptional activity by HCV-1b or 3a core protein, as well as in control cells (Fig. 4.6, a and b). Likewise, application of Akt inhibitor AktVIII at a lower, Akt-1 specific dose (58 nM) significantly decreased SRE-driven transcriptional activation by HCV core proteins either after transfection or in replicon cells. Interestingly, AktVIII inhibitor at the

higher concentration of 210 nM, which should suppress both Akt-1 and Akt-2, was much more effective in replicon cells than in transiently transfected cells (Fig. 4.5a, b). Plasmids expressing dominant negative Akt-1 or Akt-2 mutants were also used to inhibit Akt and both had a similar inhibitory effect on SRE activation (Fig. 4.5 c, d). To further confirm the role of Akt, Akt-1 and Akt-2 knock down cells were created by stably transducing Huh7 cells with a lentivirus construct containing Akt-1 or Akt-2 specific shRNA. When transfected with HCV core protein-expressing plasmids, cells with either Akt-1 or Akt-2 knocked down had significantly decreased transcriptional activity from SRE (Fig. 4.5e). These results suggest that activation of Akt-1 and Akt-2 are involved in SREBP-1 activation by HCV core.

4.5 Discussion

Clinical studies have shown that chronic HCV-3a infection leads to the development of steatosis in approximately 70% of cases. Deregulation of lipid metabolism pathways in hepatocytes is believed to be a major mechanism for the development of steatosis. The mechanism by which HCV-3a can cause steatosis is not well understood. Several studies have suggested a role for core and NS5a proteins of genotype 1b and 2a in changing lipid metabolism pathways that may contribute to steatosis observed in other genotypes.

Our previous results have shown that genotype 3a core alone is able to up-regulate transcription of FAS in an SREBP-1 dependant manner. Genotype 1b core was also able to upregulate FAS, but to a lesser extent. However, the mechanisms by which HCV core protein up-regulates FAS transcription are not clear.

In this study we sought to investigate mechanisms for the observed increase in FAS promoter activation by HCV core protein. To do this we utilized two different models: transiently transfected Huh7 cells expressing only the core protein, and stable HCV replicon expressing Huh7 cells containing either a genomic 1b replicon, or a chimeric 1b replicon containing the 3a core gene instead of the 1b core gene. Cells expressing the HCV replicon have been widely used for HCV research as they express all of the HCV proteins as a result of HCV RNA replication (Pietschmann et al., 2002).

We showed that the transcription activity from SRE was enhanced in the presence of genotype 3a core protein in comparison to genotype 1b core (Fig. 4.2, a). This would explain that HCV-3a core expression resulted in significantly higher SREBP-1c promoter activity (Fig. 4.2, c and d). In the case of HCV-1b core, it could activate transcription driven by SRE when it was expressed alone after transient transfection, but not in genomic replicon cells (Fig. 4.2, a and b). Consequently, HCV-1b core did not up-regulate SREBP-1c promoter when expressed alone or in replicon cells (Fig. 4.2, c and d). However, HCV-1b core was a stronger activator for LXRE-driven activity than HCV-3a core. This is consistent with previous studies that have observed an effect on LXR activity by HCV-1b core protein (Moriishi et al., 2007; Waris et al., 2007). These results further reinforce the notion that HCV of different genotypes has different mechanisms in causing steatosis.

SREBP-1 is translated as a larger precursor protein that is cleaved into its mature, nuclear localized and active form. Genotype 1b core and genotype 3a core have both been shown to enhance cleavage of SREBP-1 (Waris et al., 2007). In this study, we have further demonstrated an enhanced level of SREBP-1 cleavage in the presence of 3a core

protein in comparison to 1b core. The increased amount of mature SREBP-1 in core expressing cells would explain the increased transcription from the SRE as well as FAS and SREBP-1c promoters that we have observed.

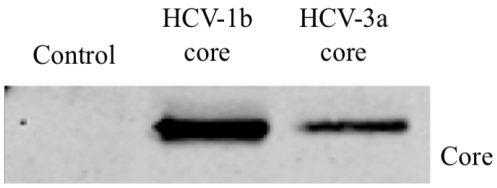
Several studies have shown that HCV affects the PI3K/Akt signaling pathway. As Akt exists in at least three isoforms that vary in function, we examined the effect HCV type 1b and 3a core protein had on Akt-1 and Akt-2 specifically. A greater effect on Ser-473 phosphorylation of Akt-1 was observed in the presence of 3a core protein in comparison to 1b core. Ser-474 phosphorylation of Akt-2 was also increased in the presence of both core proteins. Our results confirmed and expanded previous studies showing enhanced serine phosphorylation of Akt-1 by HCV core (Banerjee et al., 2008; Waris et al., 2007). Akt activity has been linked to the regulation of SREBP-1 cleavage and stability with an increased Akt activity resulting in increased SREBP-1 activity and increased expression of lipid metabolism genes (Porstmann et al., 2005). As such, we examined the role of increased Akt activity by HCV core and its effects on SREBP-1 activity. We showed that inhibiting Akt-1 and/or Akt-2by a number of methods indeed decreased SREBP-1c promoter activity as well as SREBP-1 processing and its activity as a transcriptional factor, indicating that increased activity of both Akt-1 and Akt-2 in the presence of the HCV core protein may play a role in enhancing lipid metabolism gene expression and contribute to the development of steatosis. Although statistically significant, inhibition of Akt did not completely abolish the up-regulation of SREBP-1 by HCV core and as such it is likely that other mechanisms are also involved in this process. For example, Waris *et al.* have shown that oxidative stress in cells expressing HCV core

protein can cause increased SREBP-1 and -2 processing that also contributes to the alteration of lipid metabolism in HCV infected cells.

The results of this study indicate an important role of increased Akt-1 and Akt-2 activity in mediating the effects of the HCV core protein on the expression of genes involved in lipid metabolism. In addition, this is the first study to examine the potential effects of HCV core on different isoforms of Akt. Our results further elucidate the molecular mechanisms of steatosis associated with HCV infection.

Figure 4.1. Generation and characterization of HCV chimeric replicon containing HCV-3a core. A) Expression of core protein in transiently transfected Huh7 cells. **B)** Expression of core and NS5b in 1b N replicon cells and 1b/3a core chimeric replicon cells. **C)** Transient replication of the 1b and 1b/3a core replicon in Huh7 cells.

A



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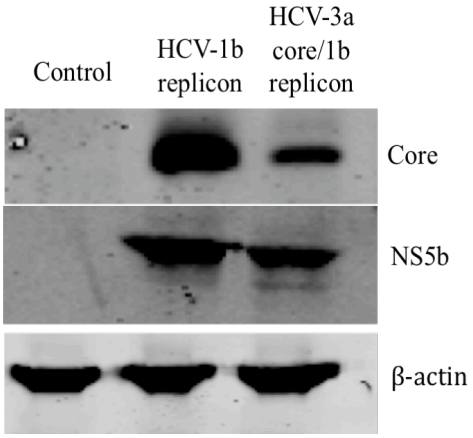

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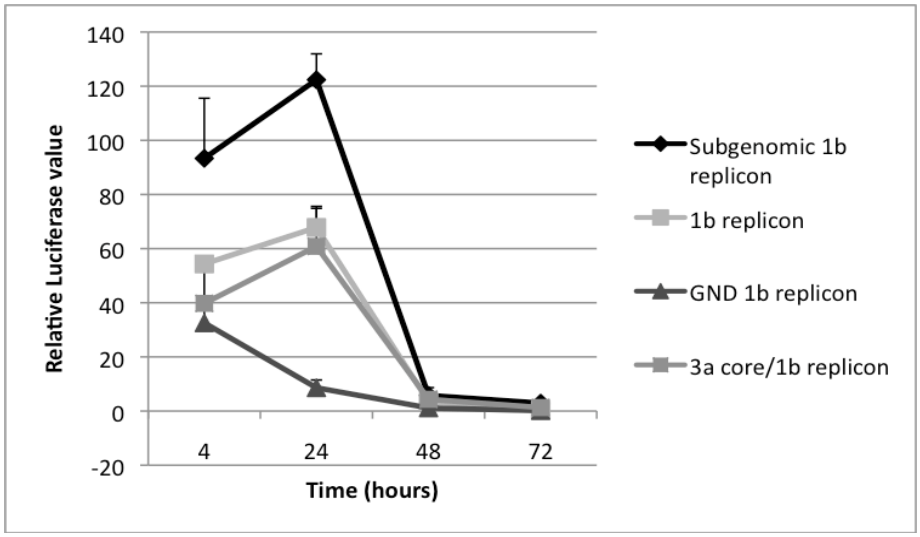
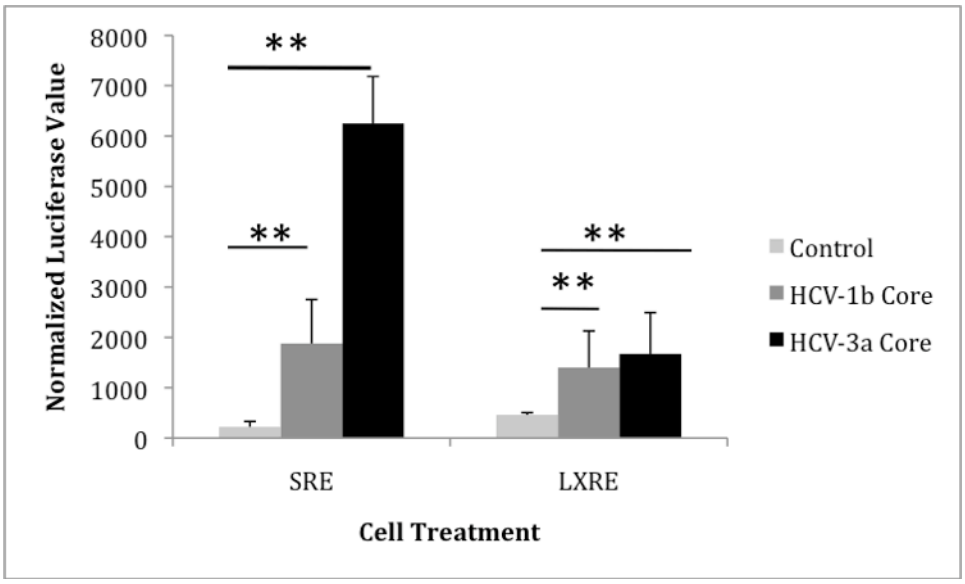


Figure 4.1

Figure 4.2. HCV-3a core protein increases transcriptional activity from the SRE and LXRE transcription factor binding motifs. A) Transcription activity from SRE and LXRE in transiently transfected Huh7 cells expressing core protein. **B)** Transcription activity from SRE and LXRE in replicon expressing cells. **C)** Deletion of SRE and LXRE promoter elements in SREBP-1c promoter in transiently transfected Huh7 cells expressing core protein. **D)** Deletion of SRE and LXRE promoter elements in SREBP-1c promoter in replicon cells. Data is representative of three separate experiments.

A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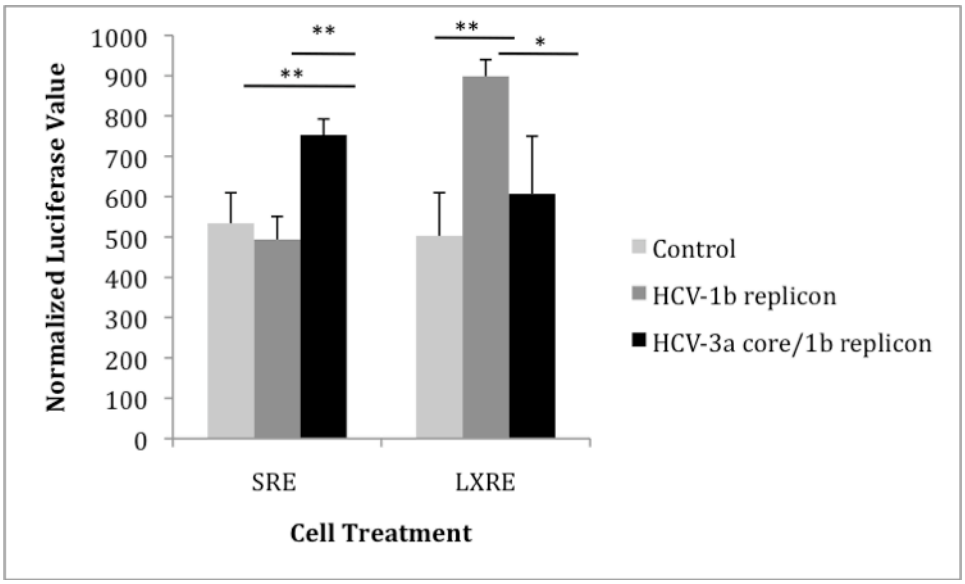
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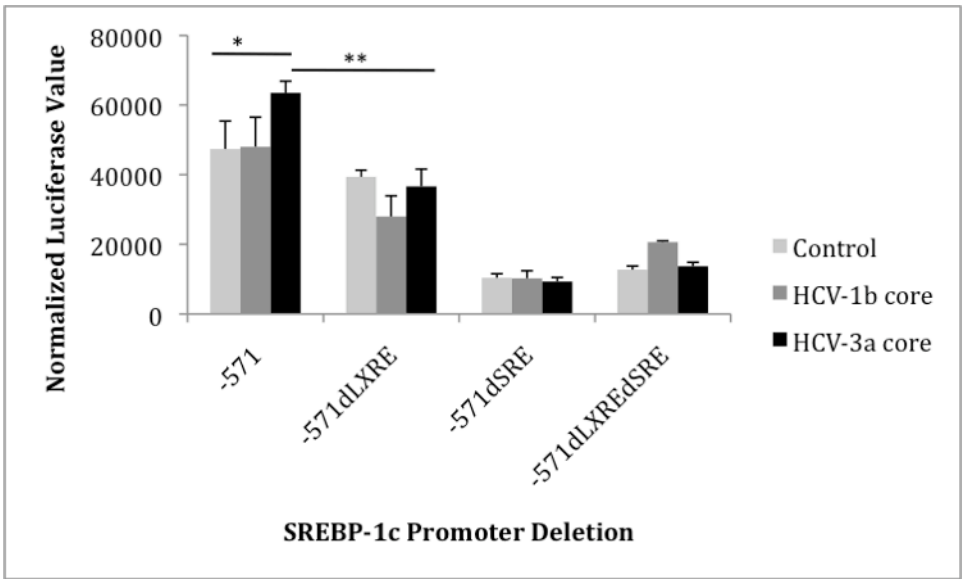
HCV-3a core/1b replicon

Figure 4.2

C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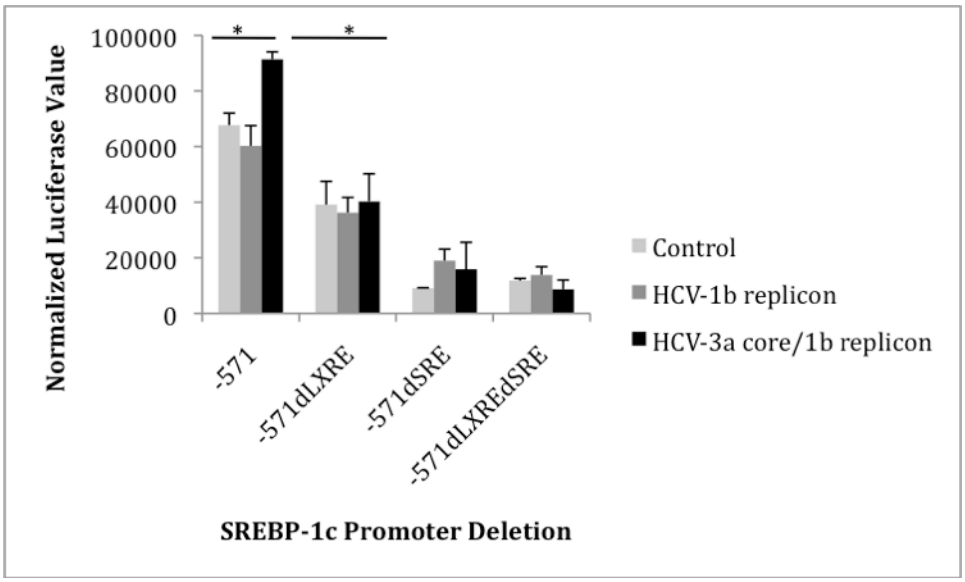
D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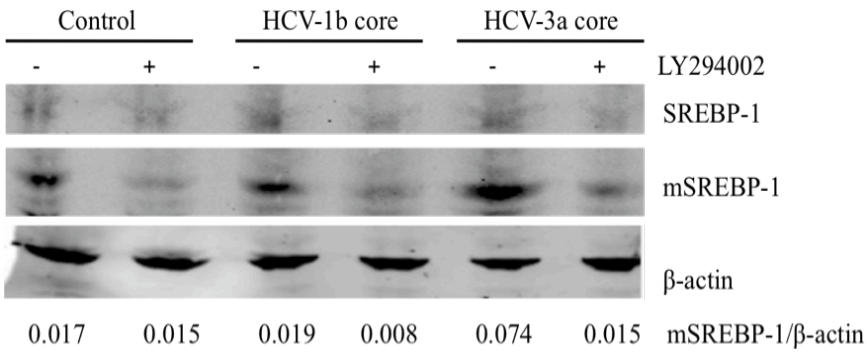
HCV-1b replicon
HCV-3a core/1b replicon

Figure 4.2

A



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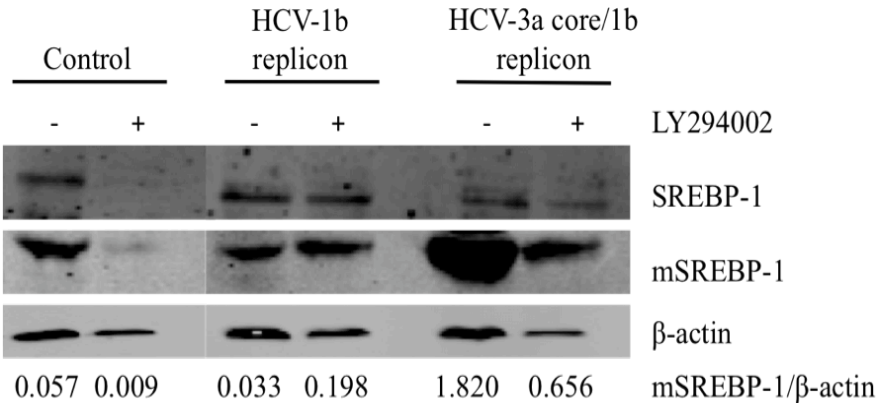
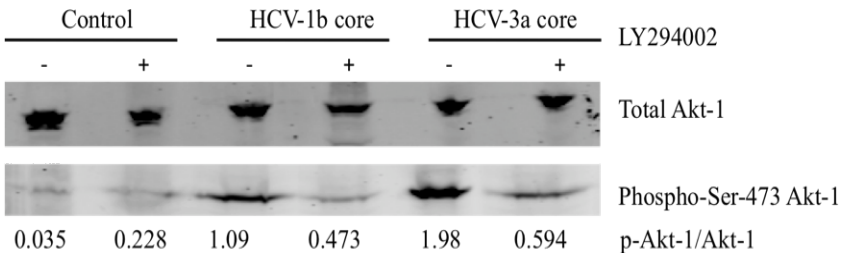


Figure 4.3. Cleavage of SREBP-1c is enhanced by HCV 3a core protein. A) Cleavage of SREBP-1 in transiently transfected Huh7 cells. **B)** Cleavage of SREBP-1 in HCV replicon cells. Cells were grown in DMEM with 1% FBS for 48 hours after transfection. 50 μM LY294002 was added 16 hours prior to harvesting. Western blots shown are representative of three separate experiments. Densitometry was determined by assessing band intensity on a LiCor Odyssey infrared scanning system and values were normalized to β-actin levels.

Figure 4.4. Phosphorylation of Akt-1 and Akt-2 are increased in the presence of HCV core. A) Endogenous Akt-1 and phosphor-Akt-1 Serine-473 in HCV core transiently transfected Huh7 cells determined by western blot. **B)** Endogenous Akt-1 and phospho-Akt-1 Serine-473 in HCV replicon expressing cells. **C)** Total Akt-2 and phospho-Akt2 Serine-474 in HCV core transiently transfected Huh7 cells. **D)** Total Akt-2 and phospho-Akt-2 Serine-474 in HCV replicon expressing cells. 50 μM LY294002 was added 16 hours prior to cell harvesting. pAkt band intensities were normalized with total Akt band intensities. Densitometry was determined using the Odyssey Imaging Software and is representative of 3 separate experiments.

A



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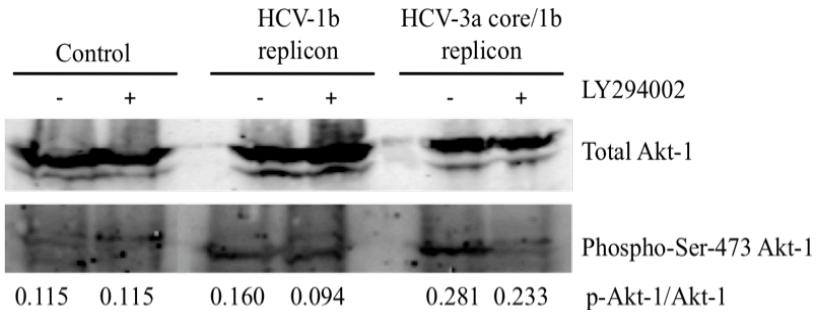

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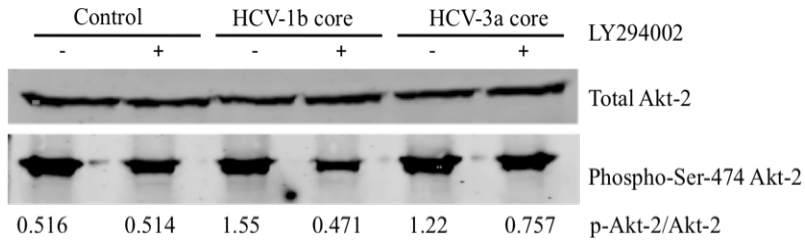


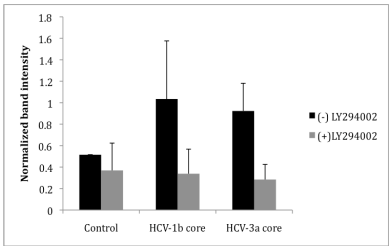


Figure 4.4

D

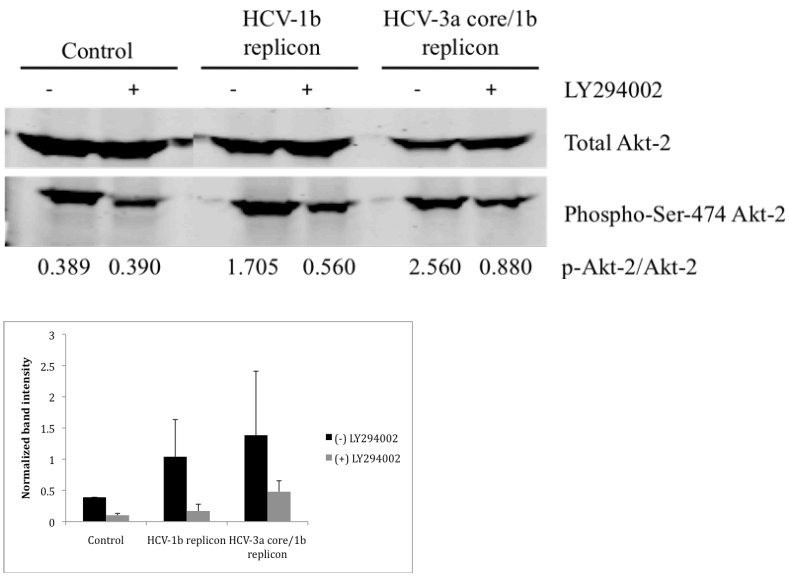
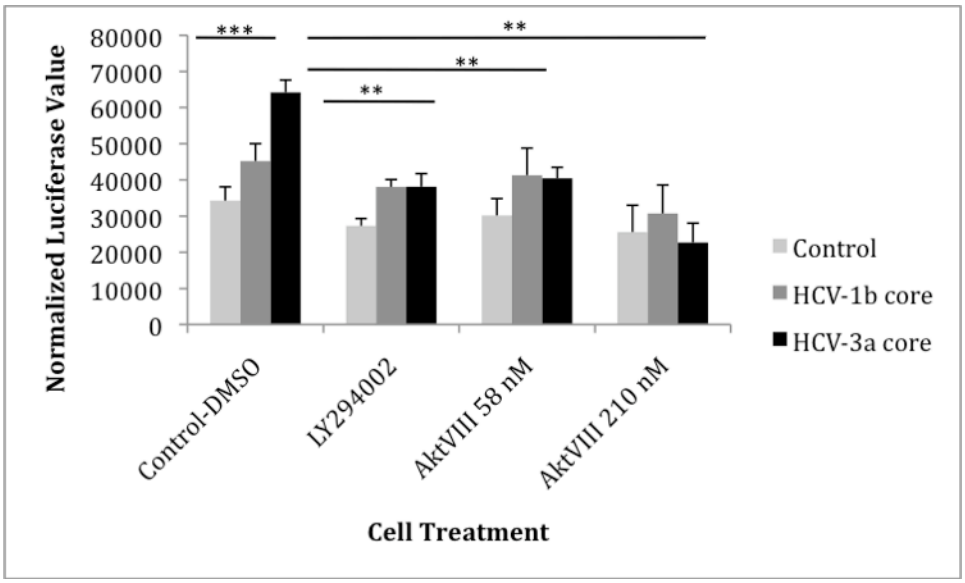

Control
HCV-1b replicon
HCV-3a core/1b replicon
- + - + - +
LY294002
Total Akt-2
Phospho-Ser-474 Akt-2
0.389 0.390 1.705 0.560 2.560 0.880
p-Akt-2/Akt-2
Normalized band intensity
3
2.5
2
1.5
1
0.5
0
(-) LY294002
(+) LY294002
Control
HCV-1b replicon
HCV-3a core/1b replicon

Figure 4.4

Figure 4.5. Inhibition of Akt causes decreased SREBP-1c transcriptional activity in the presence of HCV core protein. A) Luciferase activity from the SREBP-1c promoter in HCV core transiently transfected cells. **B)** Luciferase activity from the SREBP-1c promoter in HCV replicon expressing cells. Cells were treated with DMSO as a control. 50 μM LY294002, 58 nM AktVIII for inhibition of Akt1 or 210 nM AktVIII for inhibition of Akt1 and Akt2 were added for 16 hours prior to harvesting.

A


***
**
**
**
80000
70000
60000
50000
40000
30000
20000
10000
0
Normalized Luciferase Value
Control-DMSO
LY294002
AktVIII 58 nM
AktVIII 210 nM
Cell Treatment
Control
HCV-1b core
HCV-3a core

B

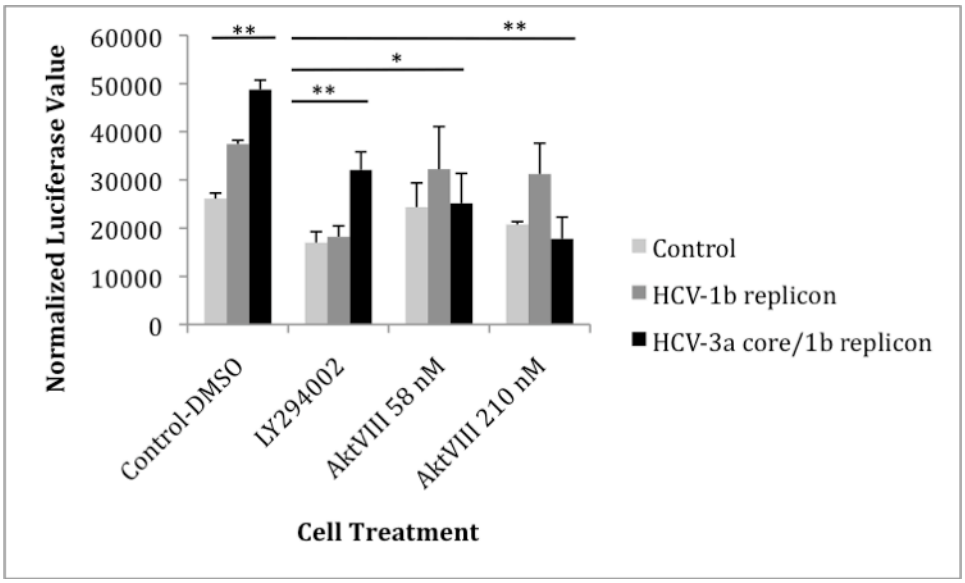
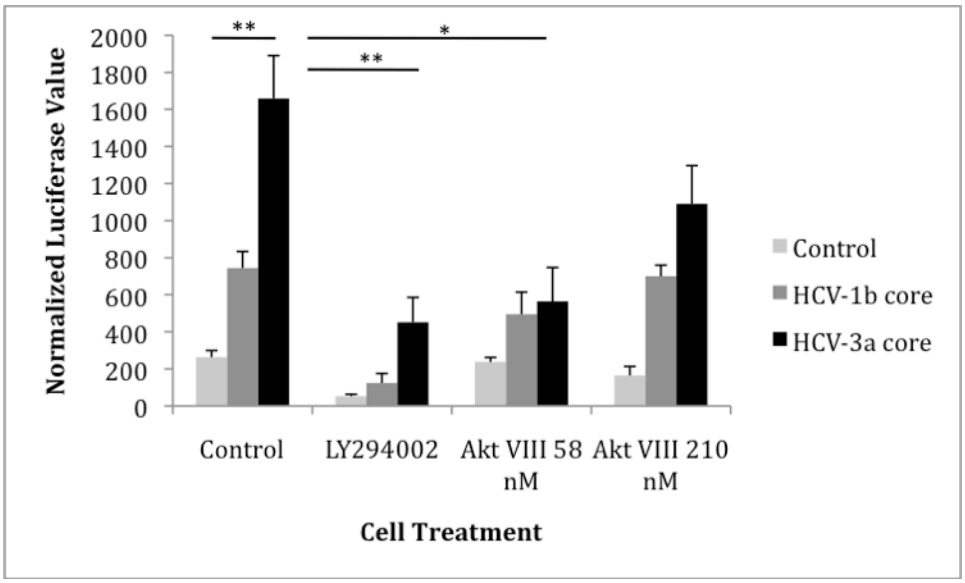

**
**
*
**
60000
50000
40000
30000
20000
10000
0
Normalized Luciferase Value
Control-DMSO
LY294002
AktVIII 58 nM
AktVIII 210 nM
Cell Treatment
Control
HCV-1b replicon
HCV-3a core/1b replicon

Figure 4.5

Figure 4.6. Inhibition of Akt activity results in decreased effect of HCV core protein on up-regulation of transcription from SRE. A) Transcription activity from SRE in transiently transfected Huh7 cells treated with LY294002 and Akt VIII chemical inhibitors of Akt. 58 nM Akt VIII inhibits Akt-1; 210 nM Akt VIII inhibits Akt-1 and Akt-2. **B)** Transcription activity from SRE in replicon expressing cells treated with LY294002 and Akt VIII chemical inhibitors of Akt. **C)** Transcription activity from SRE in transiently transfected Huh7 cells expressing dominant negative Akt-1 or Akt-2. **D)** Transcription activity from SRE in replicon cells expressing dominant negative Akt-1 or Akt-2. **E)** Generation of Akt-1 and Akt-2 knock out cells. Expression of Akt-1 or Akt-2 in Huh7 cells selected for knock down of Akt-1 or Akt-2 by an shRNA in a lentiviral vector. **F)** Transcription activity from SRE in transiently transfected cells that have Akt1 or Akt2 knocked down via stable lentivirus transduction. Cells were treated with DMSO as a control where appropriate. 50 μM LY294002, 58 nM AktVIII for inhibition of Akt-1 or 210 nM AktVIII for inhibition of Akt-1 and Akt-2 were added for 16 hours prior to harvesting. Dominant negative Akt-1 or Akt-2 expressing plasmids were transfected into cells 48 hours before harvesting.

A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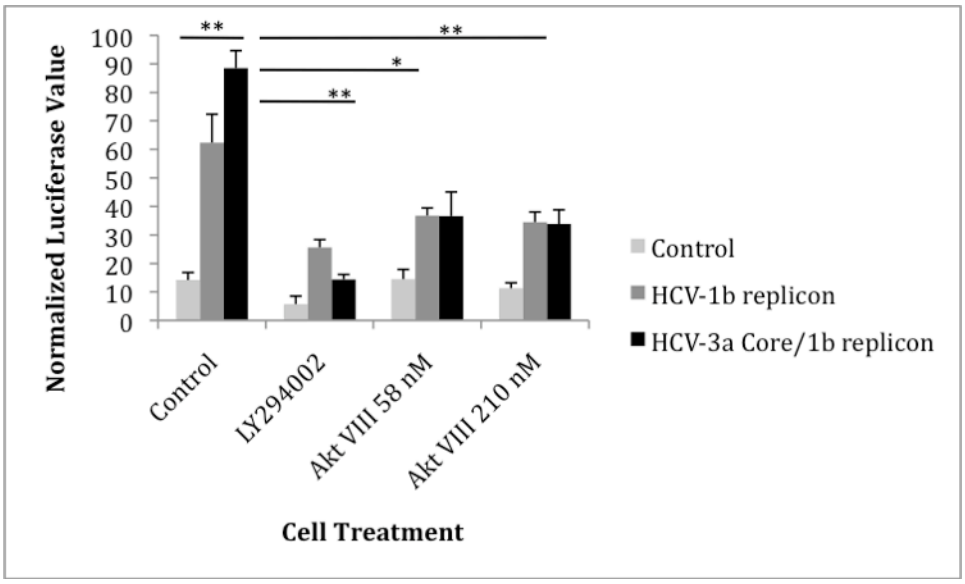
2000
1800
1600
1400
1200
1000
800
600
400
200
0
**
**
*
Control
LY294002
Akt VIII 58 nM
Akt VIII 210 nM
Cell Treatment
Control
HCV-1b core
HCV-3a core

B


Normalized Luciferase Value
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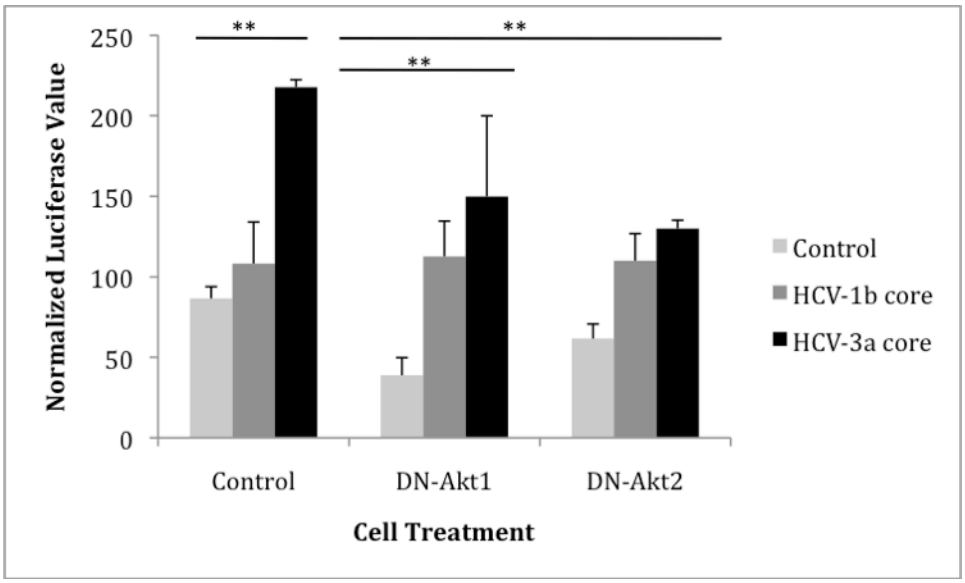
90
80
70
60
50
40
30
20
10
0
**
**
*
**
Control
LY294002
Akt VIII 58 nM
Akt VIII 210 nM
Cell Treatment
Control
HCV-1b replicon
HCV-3a Core/1b replicon

Figure 4.6

C


**
**
**
250
200
150
100
50
0
Normalized Luciferase Value
Control
DN-Akt1
DN-Akt2
Cell Treatment
Control
HCV-1b core
HCV-3a core

D

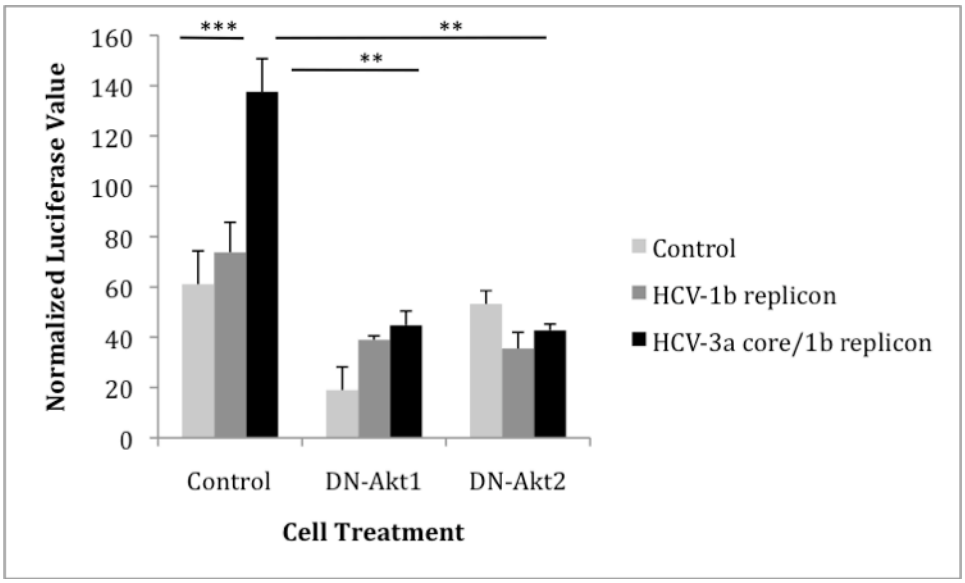
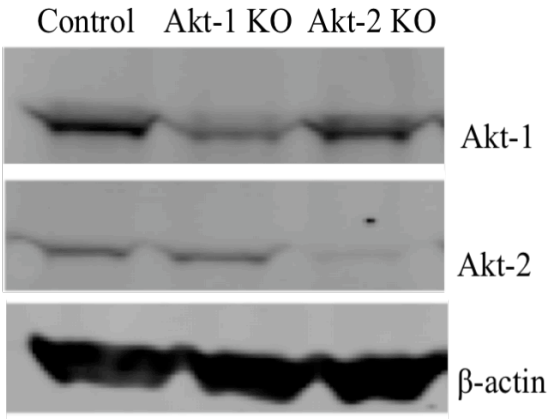

***
**
**
160
140
120
100
80
60
40
20
0
Normalized Luciferase Value
Control
DN-Akt1
DN-Akt2
Cell Treatment
Control
HCV-1b replicon
HCV-3a core/1b replicon

Figure 4.6

E


Control
Akt-1 KO
Akt-2 KO
Akt-1
Akt-2
β-actin

F

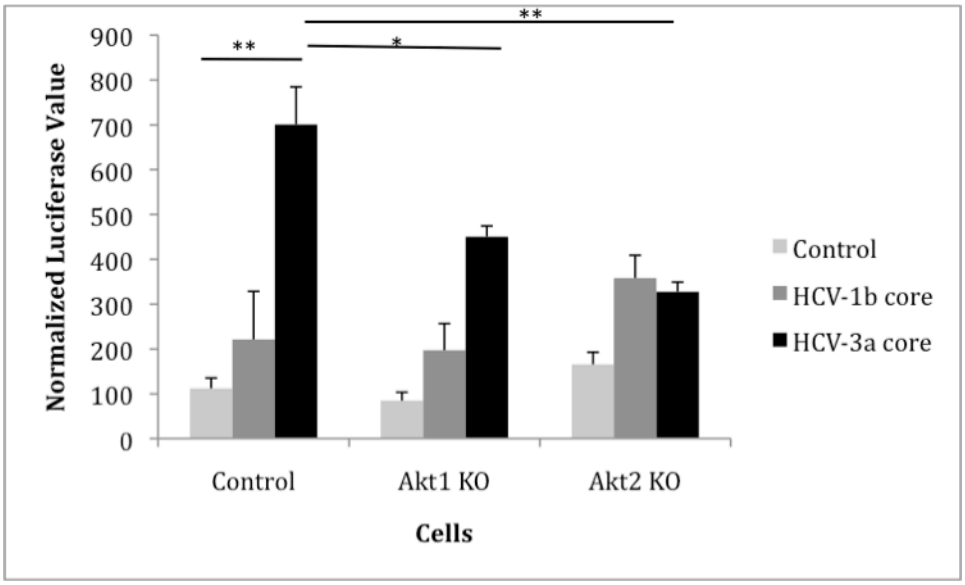

**
*
**
900
800
700
600
500
400
300
200
100
0
Normalized Luciferase Value
Control
Akt1 KO
Akt2 KO
Cells
Control
HCV-1b core
HCV-3a core

Figure 4.6

5.0 ACTIVATION OF STEROL REGULATORY ELEMENT BINDING PROTEIN-1C AND FATTY ACID SYNTHASE TRANSCRIPTION BY HEPATITIS C VIRUS NON-STRUCTURAL PROTEIN-2

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5.1 Abstract

Transcriptional factor SREBP-1c (sterol regulatory element binding protein-1c) activates the transcription of lipogenic genes, including fatty acid synthase (FAS). Hepatitis C virus (HCV) infection is often associated with lipid accumulation within the liver, known as steatosis in the clinic. The molecular mechanisms of HCV-associated steatosis are not well characterized. Here, we showed that HCV non-structural protein-2 (NS2) activated SREBP-1c transcription in human hepatic Huh-7 cells as measured by using a human SREBP-1c promoter - luciferase reporter. We further showed that sterol regulatory element (SRE) and liver X receptor element (LXRE) in the SREBP-1c promoter were involved in SREBP-1c activation by HCV NS2. Furthermore, expression of HCV NS2 resulted in up-regulation of FAS transcription. We also showed that FAS up-regulation by HCV NS2 was SREBP-1-dependent since deleting the SRE sequence in a FAS promoter and expressing a dominant-negative SREBP-1 abrogated FAS promoter up-regulation by HCV NS2. Taken together, our results suggested that HCV NS2 can up-regulate the transcription of SREBP-1c and FAS and thus is likely a contributing factor for HCV-associated steatosis.

5.2 Introduction

Sterol regulatory element binding protein-1c (SREBP-1c) is a member of the basic-helix-loop-helix-leucine zipper family of transcription factors (Horton et al., 2002;Eberle et al., 2004). SREBP-1c expression is regulated at the transcription level (Desvergne et al., 2006). For instance, liver X receptor (LXR) activates SREBP-1c transcription by binding to the LXR element (LXRE) sequences in the SREBP-1c

promoter (Yoshikawa et al., 2001;Dif et al., 2006;Tarling et al., 2004). SREBP-1c can also regulate its own transcription in a positive feed-back loop through binding to the sterol regulatory elements (SREs) in the SREBP-1c promoter (Amemiya-Kudo et al., 2000). The newly synthesized, precursor SREBP-1c protein is bound to the endoplasmic reticulum (ER). Following proteolytic digestions, the amino-terminal domain is released and transported to the nucleus as an active transcriptional factor (Brown et al., 2000). The processed, mature SREBP-1 proteins are modified by phosphorylation by protein kinases, such as mitogen-activated protein kinase (MAPK), protein kinase A (PKA), and glycogen synthase kinase-3β (Roth et al., 2000;Lu and Shyy, 2006;Punga et al., 2006;Kotzka et al., 1998). The impact of phosphorylation on the transcriptional activity of SREBP-1 is less clear. For instance, phosphorylation by MAPK appears to enhance the transcriptional activity of SREBP-1, whereas PKA phosphorylation suppresses the function of SREBP-1 (Kotzka et al., 1998;Kotzka et al., 2000;Lu and Shyy, 2006). A major function of SREBP-1c is to activate genes involved in the synthesis of fatty acid and their incorporation into triglycerides and phospholipids (Horton et al., 2002;Eberle et al., 2004). As such, abnormal higher levels of SREBP-1c will result in lipid accumulation in the liver and cause steatosis (Ferre and Foufelle, 2007). Fatty acid synthase (FAS) is a well established target gene of SREBP-1c (Latasa et al., 2000;Amemiya-Kudo et al., 2002).

Steatosis is an important clinical manifestation associated with hepatitis C virus (HCV) infection (Asselah et al., 2006). The development of steatosis in HCV infections is a complex process that likely involves both host and viral factors. As a major transcription factor for lipogenic gene expression, SREBP-1 may play a major role in this

process. For instance, HCV infection enhances the proteolytic processing of SREBP-1 (Waris et al., 2007). The expression of HCV core and non-structural protein-4B (NS4B) proteins also enhances SREBP processing and lipid accumulation (Waris et al., 2007;Kim et al., 2007;Yamaguchi et al., 2005). Our previous research showed that HCV core protein can activate FAS promoter in an SREBP-1-dependent manner (Jackel-Cram et al., 2007). Given the key role of SREBP-1c in hepatic steatosis, we investigated whether HCV NS2 protein may regulate SREBP-1c expression and thus be involved in causing steatosis. In this study, we showed that HCV NS2 protein increases SREBP-1c transcription, protein expression, and proteolytic processing. We further showed that FAS transcription is also up-regulated by HCV NS2, as a consequence of SREBP-1c activation. Our results suggested that HCV NS2 is a contributing factor for HCV-associated steatosis.

5.3 Materials and methods

5.3.1 Cloning and expression of the NS2 protein.

The coding sequence of NS2 of HCV H77 (genotype 1a) was amplified from plasmid p90/FL (Kolykhalov et al., 1997) by polymerase chain reaction (PCR) and cloned into an expression vector pEF-myc with the elongation factor-1α promoter (Invitrogen). The resultant NS2 protein had a translation initiation codon and a myc-tag at the carboxyl-terminus (Fig.1). The plasmid sequence was confirmed by DNA sequencing. To demonstrate NS2 expression, Huh-7 cells were transfected with pEF-NS2-myc and pEF-myc vector by the calcium phosphate method followed by immunoblotting as

described (Jackel-Cram et al., 2007). As shown in Fig. 5.1, a myc-tag antibody (Invitrogen) recognized a protein of approximately 23 kDa after pEF-NS2-myc transfection, but not after vector transfection, indicating the expression of the myc-tagged NS2 protein.

5.3.2 Luciferase assays

Dual luciferase assay (Promega) was done using the manufacturer's protocol. Huh-7 cells were transfected using calcium phosphate precipitation as previously described and harvested 48 hours after transfection. Huh-7 cells were transfected with HCV NS2-expressing plasmid or the vector control, together with a human SREBP-1c promoter - luciferase reporter (SREBP-1c-571-Luc-WT), kindly provided by Dr. Lefai (Dif et al., 2006). A plasmid encoding the *Renilla* luciferase gene under the control of elongation factor -1α promoter was also included in the transfections as a control for luciferase assays as described (Oem et al., 2007). To determine the involvement of LXRE and SRE, the two LXRE and two SRE sequences were eliminated by site-directed mutagenesis as described (Dif et al., 2006). The primer sequences were as follows (the mutated sequences were underlined): 5'-GAGGGCCAGAGTCCGCCAGATTCCCCGGCA- 3' and 5' -GGCGGAAGTCCGCTAGATTCCCCAACCCC- 3' for LXRE, 5' -CCATTCAGCGCCGCGAGATAAAACTCGAGCCCCC- 3' and 5' -GGCCGCGCGCGCTTATCTCATGCCCGGCCCGC- 3' for SRE, respectively. These mutant SREBP-1c promoter plasmids were confirmed by DNA sequencing and then used

in co-transfection experiment to determine the SREBP-1c promoter activity after HCV NS2 expression.

5.3.3 Immunoblotting

Transfected cells were lysed and loaded onto 10% gels for polyacrylamide gel electrophoresis. The gels were transferred onto nitrocellulose membrane, blocked in 2% milk powder in PBS, and incubated with an anti-SREBP-1 antibody (Santa Cruz Biotechnology). After washing, the blots were incubated in IRDye 680 goat anti-rabbit IgG secondary antibody (Li-Cor Biosciences). As protein loading controls, the levels of β-actin were also determined using a β-actin-specific antibody (Cell Signaling Technology). Blots were scanned on an Odyssey Infrared Imaging System (Li-Cor Biosciences). Quantification of the density ratio of the precursor and mature SREBP-1 proteins to β-actin within the same sample was performed using the Odyssey software (LI-COR Biosciences).

5.3.4 RT-PCR.

Total RNA was extracted from Huh-7 cells 48 hours after transfection with a plasmid expressing HCV NS2 or the plasmid vector by TriZol (Invitrogen) followed by a clean-up with RNeasy mini-columns (Qiagen). After digestion with an RNase-free DNase-I (Ambion), cDNA was synthesized by reverse transcription and subjected to PCR amplification using FAS-specific primers (forward: 5'-GGTCTTGAGAGATGGCTTGC-3' and reverse: 5'-AATTGGCAAAGCCGTAGTTG-3'). As a control, β-actin was amplified with primers (forward: 5'-AGCGGGAAATCGTGCGTG-3' and reverse: 5'-CAGGGTACATGGTGGTGCC-3').

The PCR products were resolved in agarose gels and analyzed by densitometry using the software Quantity One (BioRad).

5.4 Results

5.4.1 HCV NS2 protein activates the SREBP-1c promoter.

To investigate whether HCV NS2 protein can influence SREBP-1c transcription, we measured SREBP-1c promoter activity by using an SREBP-1c promoter - luciferase reporter. Huh-7 cells were transfected with HCV NS2-expressing plasmid or the vector control, together with a human SREBP-1c promoter - luciferase reporter (SREBP-1c-571-Luc-WT, Fig. 5.2a. As shown in Fig. 5.2b, expression of HCV NS2 resulted in more than four-fold induction in SREBP-1c promoter activity in comparison to vector control (Fig. 5.2b, RLU: 207±64 and 1036±143 for vector and NS2, respectively, P=0.006 as per a Student's t test), suggesting the HCV NS2 activates SREBP-1c promoter.

5.4.2 LXRE and SRE motifs in the SREBP-1c promoter are required for SREBP-1c activation by HCV NS2.

The human SREBP-1c -571 promoter sequence contains two LXREs (at positions -311/-296 and -260/-245) and two SREs (at positions -228/-218 and -127/-117) (Fig. 5.2a) (Dif et al., 2006;Tarling et al., 2004). To determine whether the LXRE and SRE sequences are involved in SREBP-1c promoter up-regulation by HCV NS2, the two LXRE and two SRE sequences were eliminated by site-directed mutagenesis. As shown in Fig. 5.2c, elimination of either LXRE or SRE motifs in the SREBP-1c promoter resulted in significantly lower SREBP-1c promoter activity in comparison to the wild-

type promoter after HCV NS2 expression (Fig. 5.2c, RLU: 1070±63, 557±31, and 432±52 for wild-type, ΔLXREs, and ΔSREs, respectively; wild-type vs. ΔLXRE, P=6.7x10^{-6}, wild-type vs. ΔSRE, P=2.2x10^{-6}). Elimination of both LXRE and SRE motifs in the SREBP-1c promoter further decreased SREBP-1c promoter activation by HCV NS2 (Fig. 5.2c, RLU: 180±21 for ΔLXRE/ΔSRE; wild-type vs. ΔLXRE/ΔSRE, P=4.9x10^{-7}, ΔLXRE vs. ΔLXRE/ΔSRE, P=3.2x10^{-6}, and ΔSRE vs. ΔLXRE/ΔSRE, P=1.5x10^{-4}). These results demonstrated that the LXRE and SRE motifs in the SREBP-1c promoter are required for SREBP-1c promoter activation by HCV NS2.

To directly demonstrate whether HCV NS2 could activate transcription driven by LXRE or SRE motifs, we used luciferase reporters containing two copies of the LXRE sequences (TGACCGGCAGTAACCC, pLXRE-Luc) (Joseph et al., 2002) or three copies of SRE sequences (ATCACCCCAC, pSRE-Luc) (Amemiya-Kudo et al., 2002), respectively. As shown in Fig. 5.2d, expression of HCV NS2 was associated with 2.8- or 5.2-fold induction of LXRE- or SRE-driven luciferase expression in comparison to vector control. Taken together, these results indicated that HCV NS2 increases SREBP-1c transcription through SRE and LXRE elements in the SREBP-1c promoter.

5.4.3 HCV NS2 enhances SREBP-1 protein expression and proteolytic cleavage.

To demonstrate whether the increased SREBP-1c transcription by HCV NS2 resulted in enhanced SREBP-1c protein expression and proteolytic cleavage, the amount of SREBP-1c protein was determined by immunoblotting using the lysates of Huh-7 cells transfected by plasmid expressing HCV NS2 or the plasmid vector control and an SREBP-1-specific antibody (Santa Cruz Biotechology). As shown in Fig. 5.2e, HCV NS2

expression was associated with increased levels of both precursor and mature SREBP-1 proteins. These results demonstrated that HCV NS2 enhances SREBP-1 protein expression and proteolytic cleavage.

5.4.4 HCV NS2 enhances FAS transcription in an SREBP-1c dependent manner.

Since FAS is a target gene of SREBP-1c, we were interested in determining whether HCV NS2 could also activate FAS transcription. For this purpose, the FAS transcript level was determined by reverse transcription (RT)-PCR. As shown in Fig. 5.3a, NS2 expression was associated with three-fold induction of FAS transcripts in comparison to vector control. This was confirmed by luciferase assay results when a FAS promoter - luciferase reporter (Swinnen et al., 1997;Jackel-Cram et al., 2007) was used to measure FAS promoter activity after NS2-expressing or vector plasmid transfection (Fig. 5.3b; RLU: 93±4 and 205±8 for vector and NS2, respectively, P=0.002). Furthermore, when the SREBP-1c binding element (SRE) was deleted from the FAS promoter, HCV NS2 was no longer able to activate FAS promoter (Fig. 5.3b; RLU: 205±8 and 16±1 for NS2+FAS-wild-type and NS2+FAS-dSRE, respectively, P=0.0007), suggesting FAS promoter up-regulation by HCV NS2 is through the SRE sequence. To further confirm the role of SREBP-1c, we used a dominant negative (DN) SREBP-1 plasmid in a co-transfection experiment as described previously (Jackel-Cram et al., 2007). As shown in Fig. 5.3c, while plasmid vector did not have an effect on FAS activation by HCV NS2, transfection of DN-SREBP-1 significantly abolished FAS activation by HCV NS2. These results indicated that HCV NS2 enhances FAS

transcription in an SREBP-1-dependent manner, probably as a consequence of SREBP-1c activation.

5.5 Discussion

The functions of HCV NS2 protein are not well understood since limited studies have been performed to understand the role of NS2 in HCV-host interactions. In this study, we showed that HCV NS2 may be a contributing factor to intracellular lipid accumulation by up-regulating the transcription of SREBP-1c through LXRE and SRE motifs in the SREBP-1c promoter. We further showed that HCV NS2 protein expression resulted in increased levels of precursor and processed SREBP-1 proteins. As a target gene of SREBP-1c, we showed that HCV NS2 can activate FAS transcription in an SREBP-1-dependent manner. These results indicate that HCV NS2 enhances SREBP-1c functional activity.

Since phosphorylation of SREBP-1 may also play a role in modulating its activity, we attempted to characterize whether HCV NS2 alters SREBP-1c phosphorylation. However, we did not detect any discernible changes in SREBP-1 phophorylation after HCV NS2 expression (data not shown; completed by Jackel-Cram). Since it has been demonstrated that HCV infection increases SREBP-1 phosphorylation (Waris et al., 2007), it would be interesting to determine which HCV proteins are responsible for the enhanced SREBP-1 phosphorylation.

The exact mechanisms of how HCV NS2 activates SREBP-1c are not clear. HCV NS2 is localized in the ER with no nuclear localization (Franck et al., 2005; Kim et al., 1999). This suggests that HCV NS2 itself is unlikely a transcriptional factor which can

directly activate SREBP-1c transcription. Further investigations are required to study this issue.

Our experimental approach was to express HCV NS2 protein in Huh-7 cells after plasmid transfection. It would be interesting to study the modulation of lipid metabolism by HCV NS2 protein in the context of HCV infection. However, this might not be readily achievable since NS2 is essential for HCV virus morphogenesis (Pietschmann et al., 2006;Yi et al., 2007).

In conclusion, our study identified a novel functional role of HCV NS2 protein in modulating lipid metabolism, which increased our understanding of the molecular mechanisms of HCV-associated steatosis.

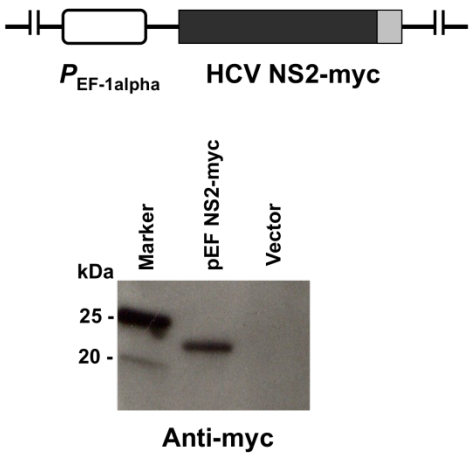
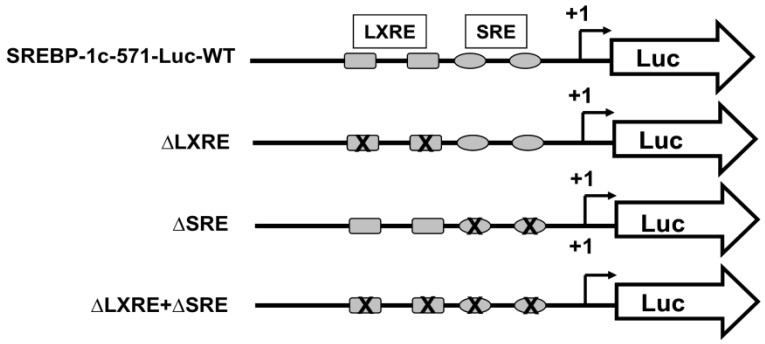


Figure 5.1. Expression of HCV NS2 protein. The coding sequence of HCV H77 NS2 was cloned into an expression vector pEF-myc, generating pEF-NS2-myc as shown in the upper panel. Expression of HCV NS2 was demonstrated in Huh-7 cells after transfection with the pEF-NS2-myc plasmid using a myc-tag antibody by immunoblotting analysis in the lower panel. Plasmid pEF-myc vector was used as a control. Experiment by Oem.

Figure 5.2. Activation of SREBP-1c by HCV NS2 protein. A) Human SREBP-1c promoter reporter constructs. A human SREBP-1c promoter (-571) - luciferase reporter as well as the locations of liver X receptor elements (LXREs) and sterol regulatory elements (SREs) in the promoter are shown. Also shown are three mutant SREBP-1c promoter reporters without the two LXREs, the two SREs, or both. **B)** Activation of SREBP-1c promoter by HCV NS2. Huh-7 cells were transfected with HCV NS2-expressing plasmid or the plasmid vector, together with an SREBP-1c promoter - luciferase reporter. Luciferase assay was performed 48 hours after transfection to determine the SREBP-1c promoter activity. The statistical differences between the samples were demonstrated as ** if $p \leq 0.01$. **C)** Activation of SREBP-1c promoter by HCV NS2 requires LXRE and SRE sequences. Huh-7 cells were transfected with HCV NS2-expressing plasmid or the plasmid vector, together with wild-type or mutant SREBP-1c promoter - luciferase reporters. Luciferase assay was performed 48 hours after transfection. The statistical differences were demonstrated as *** if $p \leq 0.001$. **D)** Activation of LXRE- and SRE-driven transcription by HCV NS2. Huh-7 cells were transfected with a plasmid expressing HCV NS2 or the plasmid vector, together with pLXRE-Luc or pSRE-Luc. Luciferase assay was performed 48 hours after transfection. The statistical differences were demonstrated as ** if $p \leq 0.01$ or *** if $p \leq 0.001$. **E)** HCV NS2 enhances SREBP-1c protein expression and proteolytic processing. Huh-7 cells were transfected with HCV NS2-expressing plasmid or the plasmid vector. In the upper panel, the expression of myc-tagged NS2 protein was confirmed by immunoblotting using a myc-tag antibody. The precursor and mature SREBP-1 protein levels were determined using an SREBP-1-specific antibody. The level of β-actin was

demonstrated by a β-actin antibody. The intensity of the protein bands quantified by the Odyssey Infrared Imaging System (LI-COR Biosciences) was given under the corresponding protein bands. The relative levels of precursor and mature SREBP-1 after vector or NS2-expressing plasmid transfection were presented as the ratio of SREBP-1 proteins to β-actin in a graph in the lower panel. Experiments completed by Jackel-Cram.

A


LXRE
SRE
+1
SREBP-1c-571-Luc-WT
Luc
ΔLXRE
ΔSRE
ΔLXRE+ΔSRE

B

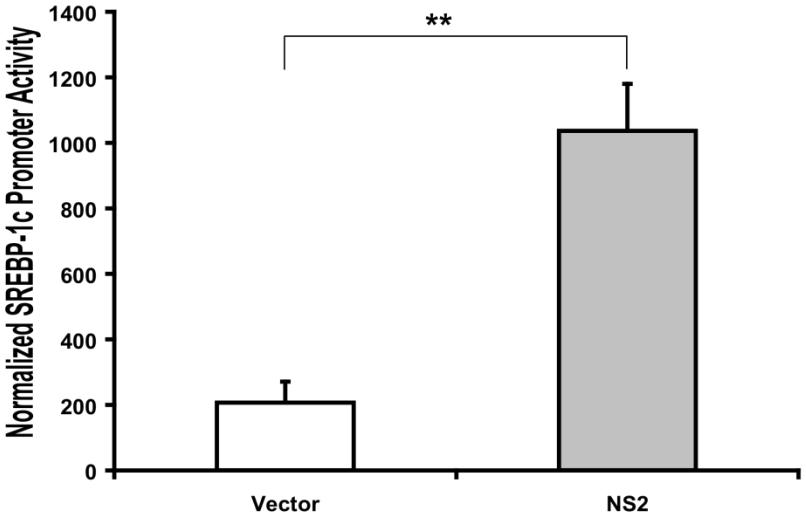
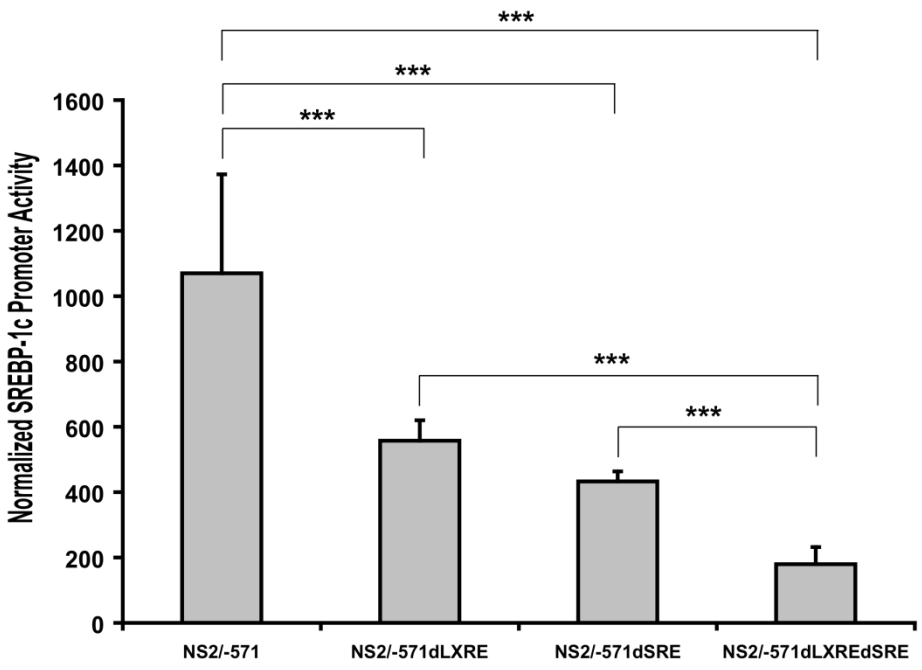

**
Normalized SREBP-1c Promoter Activity
1400
1200
1000
800
600
400
200
0
Vector
NS2

Figure 5.2

C


***
***
***
***
***
1600
1400
1200
1000
800
600
400
200
0
Normalized SREBP-1c Promoter Activity
NS2/-571
NS2/-571dLXRE
NS2/-571dSRE
NS2/-571dLXREdSRE

D

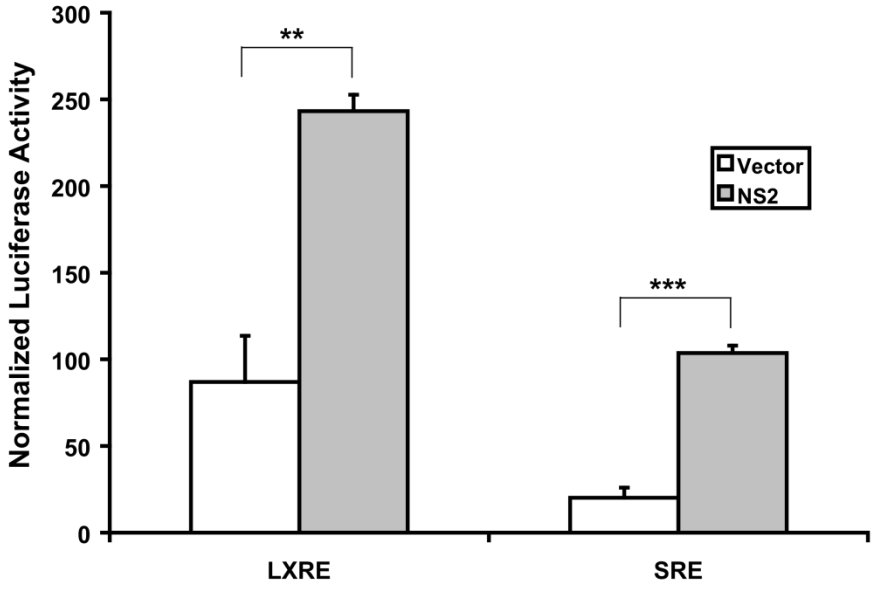

**
***
300
250
200
150
100
50
0
Normalized Luciferase Activity
Vector
NS2
LXRE
SRE

Figure 5.2

E

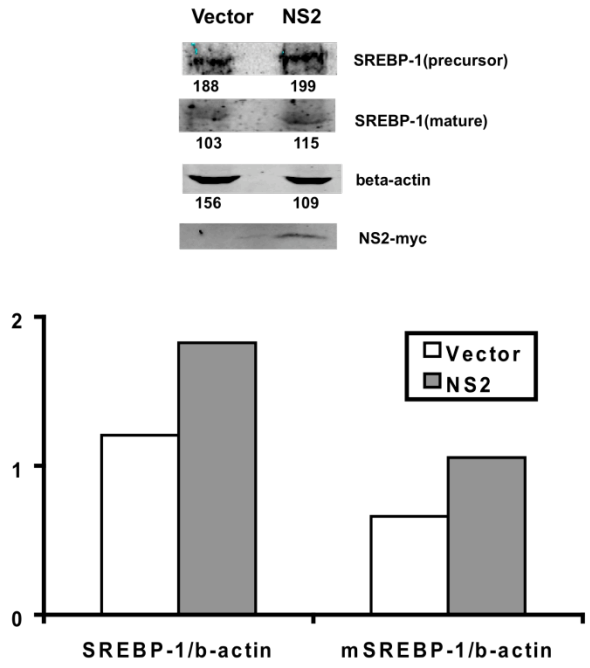
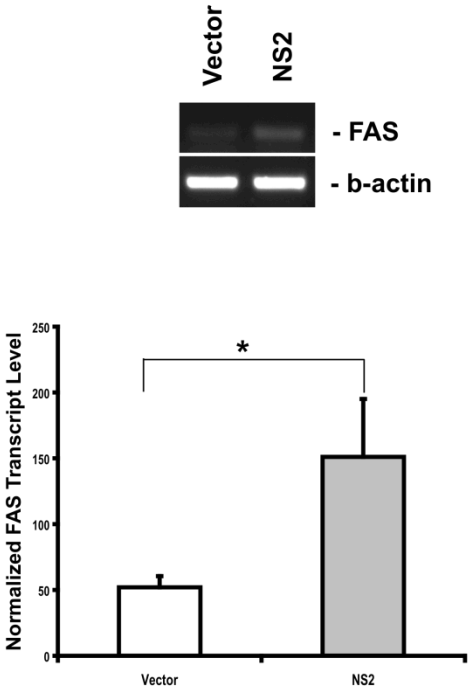

Vector
NS2
SREBP-1(precursor)
188
199
SREBP-1(mature)
103
115
beta-actin
156
109
NS2-myc
2
1
0
Vector
NS2
SREBP-1/b-actin
mSREBP-1/b-actin

Figure 5.2

Figure 5.3. Activation of fatty acid synthase (FAS) transcription through SREBP-1c by HCV NS2 protein. A) Activation of FAS transcription by HCV NS2. FAS transcript levels were determined by RT-PCR using FAS-specific primers in Huh-7 cells 48 hours after transfection with a plasmid expressing HCV NS2 or the vector control. As a control, the level of β-actin was also determined. The PCR products were analyzed by agarose gel electrophoresis in the upper panel followed by densitometry analysis shown in a graph in the lower panel. The statistical differences were demonstrated as * if $p \leq 0.05$. **B)** Activation of FAS promoter by HCV NS2. Huh-7 cells were transfected with HCV NS2-expressing plasmid or the vector control, together with wild-type or SRE-deleted FAS promoter - luciferase reporters. Luciferase assay was performed 48 hours after transfection to determine the FAS promoter activity. The statistical differences were demonstrated as ** if $p \leq 0.01$ or *** if $p \leq 0.001$. **C)** Dominant-negative (DN) SREBP-1 abrogated FAS activation by HCV NS2. Huh-7 cells were transfected with a plasmid expressing HCV NS2 or the plasmid vector, a wild-type FAS promoter - luciferase reporter, together with pcDNA3.1(+) vector or a plasmid expressing DN-SREBP-1. Luciferase assay was performed 48 hours after transfection. The statistical differences were demonstrated as * if $p \leq 0.05$ or *NS* for not significant. Experiments by Oem.

A


Vector
NS2
- FAS
- b-actin
Normalized FAS Transcript Level
250
200
150
100
50
0
*
Vector
NS2

B

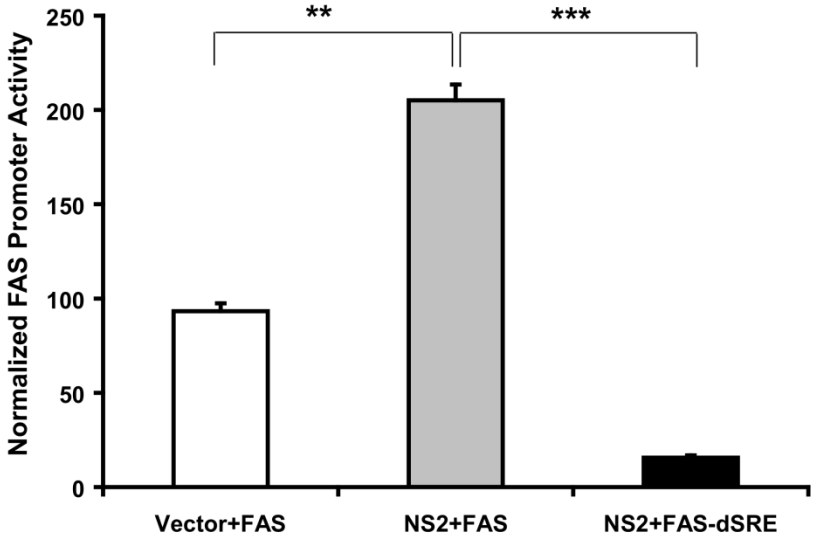

Normalized FAS Promoter Activity
250
200
150
100
50
0
**
***
Vector+FAS
NS2+FAS
NS2+FAS-dSRE

Figure 5.3

C

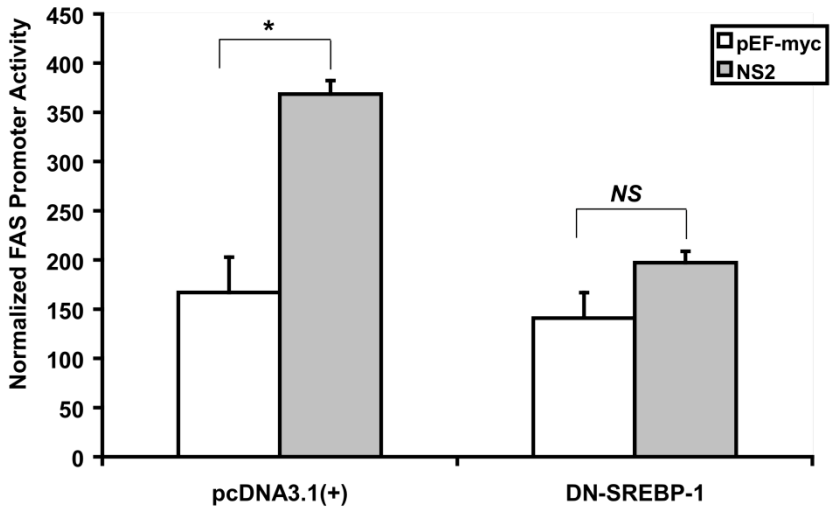

Normalized FAS Promoter Activity
450
400
350
300
250
200
150
100
50
0
*
NS
pEF-myc
NS2
pcDNA3.1(+)
DN-SREBP-1

Figure 5.3

6.0 DISCUSSION AND CONCLUSIONS

Infection with HCV is very serious and life threatening with limited treatment options. The virus primarily infects hepatocytes and it is one of the leading causes of liver disease in the world. One of the primary roles of the liver is the metabolism of triglycerides and cholesterol and, as such, it is unsurprising that HCV infection alters lipid metabolism processes within hepatocytes. A clear association was revealed between the development of steatosis and infection with HCV (Mihm et al., 1997). Approximately 50% of HCV infected individuals have steatosis, compared with only 20% of the uninfected population. Steatosis, although largely asymptomatic, is considered a precursor to the development of further liver disease such as hepatitis, fibrosis and cirrhosis. The presence of steatosis is also known to decrease the chance of responding successfully to interferon therapy. Examining the mechanisms by which HCV causes steatosis could provide important information on how to slow the progression of liver damage or increase treatment outcomes. Interestingly, infection with genotype 3 HCV appears to be more strongly associated with the development of steatosis and it may have a direct cytopathic effect on infected hepatocytes that results in the development of steatosis (Rubbia-Brandt et al., 2000). Exactly how and why there is a difference between genotype 3 and other genotypes is not fully understood. As such, determining the mechanisms for this difference was one of the main objectives of this research.

6.1 Up-regulation of fatty acid synthase promoter by hepatitis C virus core protein: genotype 3a has a stronger effect than genotype 1b-core

Previous HCV research has focused on genotype 1 infection due to the increased prevalence of this genotype and the difficulty in successfully treating patients infected with it. Very little information was available on genotype 3 infection, especially any molecular aspects of the virus that could account for the differences observed in treatment response and association with steatosis. Furthermore, very little was known about how HCV caused changes in lipid metabolism. It was clear, however, that the core protein was the likely culprit, as it was known to associate with lipid droplets in hepatocytes (Barba et al., 1997). Core protein could also bind to apolipoprotein AII and decrease the activity of MTP, thereby inhibiting secretion of VLDL (Sabile et al., 1999; Perlemuter et al., 2002). As such, it was necessary to first characterize the genotype 3a core protein to ascertain whether it acted in a similar manner to genotype 1 core protein. The genotype 3a core gene was cloned from infected patient serum and into an expression plasmid for further examination. Subcellular localization using confocal microscopy showed a distinct localization of core protein of both genotypes on lipid droplets in transiently transfected Huh-7 cells. This was the first study to show that the genotype 3a core protein was also able to localize on lipid droplets, an important first step in characterizing the genotype 3a core protein. Although the methodologies used were not sensitive enough to reveal a difference, research done by other groups has subsequently shown that the core protein is able to change the distribution of lipid droplets within hepatocytes (Boulant et al., 2008). Core protein is also able to increase the amount and size of lipid

droplets, and this effect is even more pronounced in the presence of genotype 3a core protein (Abid et al., 2005; Piodi et al., 2008; Jhaveri et al., 2008).

Since studies with the genotype 1 core protein had identified the sequence motifs responsible for lipid droplet binding (Hope and McLauchlan, 2000; Hope et al., 2002; McLauchlan et al., 2002; Suzuki et al., 2005), it was necessary to determine if the same sequence motifs were responsible for localization of the 3a core protein on lipid droplets. Site- directed mutatgenesis directed at the proline knot region, which mimics a lipid droplet localization motif in plant oleosin protein, was able to reduce lipid droplet localization of both 1b and 3a core proteins. Deletion of the YATG sequence in 1b core or FATG in 3a core was also able to reduce lipid droplet localization. These results suggest that similar sequences are involved in lipid droplet localization of both proteins.

In order to determine if there were any functional differences between the genotype 1 and genotype 3a core proteins that influenced the lipid pathways of metabolism, the transcriptional activation of FAS was examined. A previous study by Su *et al.* had discovered a potential role for SREBP in HCV pathogenesis and replication during chimpanzee infection using microarray analysis (Su et al., 2002). FAS transcription is mediated by SREBP and it is one of the key enzymes responsible for the production of triglycerides in hepatocytes. Therefore, up-regulation of the transcription of FAS could explain how HCV infection causes increased lipid in hepatocytes. Using a luciferase reporter assay with the FAS promoter, an almost two-fold increase in FAS transcription was observed in the presence of the 3a core protein in comparison to 1b core. Unsurprisingly, 1b core also increased FAS transcriptional activity, but not to the same extent as 3a core protein. In addition, a dominant negative mutant of SREBP-1 was

able to completely inhibit the effect of the core proteins on FAS promoter activity, validating the hypothesis that the effect HCV core has on lipid metabolism is mediated via increased SREBP-1 activity. This was the first study to show a potential mechanism for the differences observed between genotype 1b and 3a core proteins in causing increased triglyceride accumulation and steatosis.

To further examine how FAS promoter activity was being up-regulated by the core protein, it was necessary to determine if the lipid droplet localization of the core proteins played a role. The mutant 1b and 3a core proteins were used in the same luciferase reporter assay for FAS promoter activity. There were no significant differences observed with the proline knot mutations, but deletion of the FATG sequence in 3a core was able to significantly decrease FAS up-regulation. The phenylalanine residue present in this sequence in 3a core is a tyrosine residue in 1b core and it is not conserved in the 3a core gene. Site-directed mutatgenesis was used to create a 3a core protein containing the tyrosine residue in place of the wild type phenylalanine. This mutant reduced the up-regulation effect on FAS in comparison to the wild type 3a core protein, suggesting that this amino acid could be very important to the development of steatosis in HCV infection with genotype 3a. Subsequently, Hourioux *et al*. also showed that this particular phenylalanine residue in the 3a core protein was able to induce triglyceride accumulation in hepatocytes, confirming its relevance in the development of steatosis by genotype 3a core (Hourioux et al., 2007). Other groups have also examined whether specific polymorphisms in the genotype 3a core protein could be associated with steatosis. Jhaveri *et al*. identified two residues at positions 182 and 186 in the 3a core protein that correlated with the presence of steatosis in HCV genotype 3a infected

patients. When these residues were mutated, increased lipid levels were no longer observed in an *in vitro* model (Jhaveri et al., 2008). However, Piodi *et al*. was unable to identify a specific amino acid residue(s) that could explain the development of steatosis in genotype 3a infection. Both studies compared patients with and without steatosis infected with genotype 3 to try to explain why some individuals with genotype 3 infection do not develop steatosis. Their studies suggested that the development of steatosis may not be as simple as a polymorphism at one or more amino acid residues in the core proteins (Piodi et al., 2008). This is not surprising because increased SREBP-1 and/or FAS activity does not necessarily result in the development of steatosis because it is a complex process. Host factors may still play a role in the development of steatosis, and other HCV proteins may also be involved in altering lipid metabolism.

6.2 Hepatitis C virus genotype 3a core protein enhances sterol response element binding protein-1 transcription and cleavage via increased protein kinase B/Akt activity

The next objective of this research was to further examine the role of SREBP-1 in mediating the effects of genotype 1b and 3a core proteins on lipid metabolism. To do this, a HCV chimeric replicon expressing cell line was developed that contained the 3a core gene in a 1b HCV backbone. Replicon cell lines represent an important *in vitro* model for the study of HCV replication and pathogenesis. They contain the positive sense HCV RNA genome that stably replicates and produces the negative strand genome as well as all of the HCV proteins. However, with the exception of the JFH-1 HCV replicon and its derivatives, the replicon cell lines do not produce infectious viral

particles. The replicon system provides a more accurate representation of infection within cell culture than transient transfection experiments that over-express one or more of the HCV proteins. Replicon cell lines were used in the second study in order to determine how the presence of all of the HCV proteins could influence the cell signaling pathways being examined to ensure that the results obtained were valid and accurate. In addition, similar experiments employing transient transfections when only the core proteins were expressed confirmed that the core protein was the primary mediator of the effects observed.

As previously discussed, SREBP-1 is activated when sterol levels are low or when insulin is present. Once cleaved, it is translocated into the nucleus and binds to the Sterol Response Element (SRE) sequence TCACNCCAC in its own promoter as well as in many different genes involved in lipid metabolism. A luciferase reporter under the control of the SRE was used to determine the activity from the SRE in the presence of 1b or 3a core protein. Consistent with FAS transcriptional activity, an increased level of activity from the SRE was observed in the presence of 3a core proteins in comparison to 1b core in both transient transfections and replicon cells. In contrast, no significant difference was observed using the Liver X Receptor Element (LXRE) in the same experiments. LXR is another transcription factor that controls the expression of various genes involved in lipid metabolism, including SREBP-1, and it may act together with SREBP-1 upon insulin activation (Chen et al., 2004). To further confirm the role of SRE, the SREBP-1 promoter was used in luciferase assays with the core protein. Upon deletion of the SRE or LXRE, or both binding elements, transcriptional activity was decreased, suggesting that although both binding elements play a role in transcriptional

activation in the presence of core, only SREBP-1 activity is significantly increased by 3a core protein. Moriishi *et al.* also observed an involvement of LXR in increasing SREBP-1c promoter activity in the presence of HCV 1b core protein (Moriishi et al., 2007). Interestingly, another study that examined the level of SREBP-1c mRNA in liver biopsies from chronically infected HCV patients concluded that there was no significant difference in SREBP-1c mRNA levels between infected and uninfected liver samples. One possible explanation for the discrepancy between this study and other *in vitro* and chimpanzee studies that have shown increased SREBP-1 is that this study examined chronically infected liver tissue, rather than those undergoing acute infection. It is possible that the influence of HCV on lipid metabolism is different during different stages of infection. Regardless, FAS mRNA levels were higher in HCV infected patients with steatosis and insulin resistance, suggesting that FAS up-regulation may be important throughout HCV infection and that other mechanisms may regulate FAS during chronic infection (McPherson et al., 2008).

Although increased activity from the SRE can indicate increased activity of SREBP-1, it was necessary to examine how this increased activity of SREBP-1 was occurring. Since processing of SREBP-1 into its mature form is critical for its role as a transcription factor, it was important to examine the processing of SREBP-1 in the presence of the core proteins. A dramatic difference was observed in the amount of processed or mature SREBP-1 in the presence of 3a core protein in comparison to 1b core processing. This result suggests that the mechanism by which 3a core activates SREBP-1 activity is via increasing the processing or stability of mature SREBP-1. Waris *et al*.

observed a similar effect on SREBP using both the JFH-1 cell culture system and the 3a core protein alone (Waris et al., 2007).

Despite this information, the exact mechanism for the increased activity of SREBP-1 in the presence of core proteins remains unclear. Recent studies have suggested that the processing of SREBP-1 may be regulated partially by Akt, an important cellular signaling kinase involved in insulin signaling and gluconoegenesis. Inhibition of Akt using a chemical inhibitor, LY294002, also inhibited processing and activity of SREBP-1 (Fleischmann and Iynedjian, 2000; Porstmann et al., 2005). Several studies have shown an increased activity of Akt in the presence of core protein or in replicon cells; however, others have shown a decrease on Akt activity (Mannova and Beretta, 2005; Waris and Siddiqui, 2005; Waris et al., 2007; Yao et al., 2004; Kawaguchi et al., 2004; Miyamoto et al., 2007). Our study showed an increased serine phosphorylation of Akt-1 in the presence of either 1b core or 3a core. We were also interested in the potential role of Akt-2 in the increased processing of SREBP-1. Akt-2 is putatively involved primarily in insulin signaling (Dummler et al., 2006). Akt-2 phosphorylation of serine-474 was consistently, but not significantly, increased in the presence of the 1b or 3a core protein. Inhibition of Akt-2 by a dominant negative mutant was also able to decrease SRE activity. As such, Akt-2 may be important in mediating the effect of the core protein on lipid metabolism, but more research is needed to determine its exact role. This is the first study to suggest a role for Akt2 in the pathogenesis of HCV.

The next part of this study was to determine if inhibition of Akt-1 or Akt-2 could impair SREBP-1 activation by the core proteins. To measure this we used the luciferase

reporter assay with the SRE binding element. We inhibited Akt with the chemical inhibitor LY294002 or the isoforms specific inhibitor Akt VIII. We also used dominant negative mutants of Akt-1 or Akt-2 and knock out cell lines for Akt-1 or Akt-2. All of these approaches resulted in the inhibition of SREBP-1 activity to some extent, with the LY294002 inhibitor having the greatest effect. The impairment of SREBP-1 activity was not complete though, and likely other mechanisms such as ER or oxidative stress in the presence of HCV can also influence SREBP-1 activity (Waris et al., 2007).

This study identified one mechanism of how the 1b and 3a core proteins are able to enhance triglyceride synthesis via increased SREBP-1 activity. The enhancement of SREBP-1 activity *in vitro* could help to explain how HCV 1b, and especially HCV 3a, are contributing to the development of steatosis *in vivo*. It would be interesting to repeat this work using the JFH-1 infectious cell culture system to confirm that the same effect on lipid metabolism occurs during full HCV infection in cell culture. As JFH-1 is a genotype 2a virus, chimeric viruses containing 1b or 3a genes would need to be developed and characterized. Several studies have shown that this is possible, although virus production is much less than wild type JFH-1. This could become a problem when attempting to compare the effects of different chimeric viruses on lipid metabolism (Pietschmann et al., 2006). However, one group has successfully developed a chimeric virus that produces high levels of infectious virus expressing 2a or 3a core proteins and could be useful for these studies (Gottwein et al., 2007).

6.3 Activation of sterol regulatory element-binding protein 1c and fatty acid synthase transcription by hepatitis C virus non-structural protein 2

In the last part of this study, we hypothesized that other HCV proteins might be involved in altering lipid metabolism during infection. Miyanari *et al.* showed that core proteins recruit non-structural proteins (NS3, NS4ab, NS5a, NS5b) to the surface of lipid droplets for assembly and production of infectious virus particles, suggesting that non-structural proteins may also play a role in altering lipid metabolism. Recently it has been determined that HCV NS2 protein is critical for the assembly and production of infectious virus (Jones et al., 2007; Jirasko et al., 2008). Very little is known about whether or not NS2 plays a role in HCV pathogenesis. As such, we investigated whether the genotype 1b NS2 protein could influence the same lipid metabolism pathways that the core protein was able to.

In order to examine the role of NS2 in lipid metabolism, we first looked at its ability to activate the promoter of SREBP-1c using the same luciferase reporter system as used previously. Surprisingly, the NS2 protein was able to significantly enhance transcription from the SREBP-1c promoter compared to the control. In addition, deletion of the SRE and LXRE binding elements in the SREBP-1c promoter significantly inhibited the ability of NS2 to increase the promoter activity of SREBP-1c. This was the first evidence that had been presented on a role of NS2 protein in the enhancement of lipid metabolism *in vitro.*

The increased activity of the SREBP-1c promoter suggested that the level of SREBP-1 protein and its mature form could also be increased in the presence of NS2. As such, we examined the level of precursor and mature SREBP-1 protein in the presence of

NS2 using immunoblotting. We found that the level of both the precursor and mature SREBP-1 was increased by NS2. This increase could explain the increased transcriptional activity from the SREBP-1c promoter.

As previously mentioned, FAS transcription is regulated by SREBP-1. We examined the amount of FAS mRNA in cells expressing NS2 and we found that the level of FAS mRNA was significantly enhanced. In addition, luciferase reporter assays using the FAS promoter revealed an enhanced level of activity from the FAS promoter in the presence of NS2, confirming the RT-PCR results. This effect on FAS transcription was determined to be dependent on the SREBP-1.

This study has demonstrated a novel role for NS2 protein in altering lipid metabolism by enhancing SREBP-1c activity in a similar manner to the core protein. Both NS5a and NS4a have been implicated in altering lipid metabolism *in vitro* and so it is not surprising that NS2 could have a similar effect on lipid metabolism that may be indirect or non-specific (Park et al., 2009; Shi et al., 2002). NS2 localizes to the ER membrane along with the core protein and several other HCV proteins. SREBP-1 is bound to the ER membrane until it is cleaved and enters the nucleus to act as a transcription factor. The localization of the HCV proteins to the ER could be positively influencing the cleavage of SREBP-1 regardless of the levels of lipid in the cells. More studies will be necessary to determine if the HCV proteins are causing ER stress or if they are activating the regulators of SREBP-1 cleavage: SCAP, Insig, S1P and S2P. Several studies have shown other HCV non-structural proteins as having a role in altering lipid metabolism (Park et al., 2009; Shi et al., 2002; Waris et al., 2007). The involvement of multiple HCV proteins in altering lipid metabolism pathways suggests that these

pathways are very important to the virus life cycle and HCV has evolved multiple mechanisms to exploit these pathways to use to it's advantage.

6.4 General conclusions:

1. HCV enhances lipid production *in vitro* via increased activity of SREBP-1. This effect may partially explain how HCV infection causes the development of steatosis during chronic infection.

2. HCV genotype 3a core protein has a greater effect on lipid metabolism than genotype 1b *in vitro*, suggesting that the 3a core protein may be responsible for the development of steatosis in genotype 3a-infected individuals.

3. The NS2 protein may also play a role in the development of steatosis during HCV infection.

Throughout the course of this research, it has become more and more apparent that alteration of lipid metabolism is very important for the life cycle hepatitis C virus. HCV appears to exploit the VLDL assembly and secretion pathway for its own assembly and secretion, thereby producing virus particles bound to host proteins that can evade the immune system and utilize host lipoprotein receptors for entry and infection (Gastaminza et al., 2008; Burlone and Budkowska, 2009; Huang et al., 2007). Furthermore, inhibition of FAS activity using chemical inhibitors can reduce HCV production and entry,

suggesting that the enhancement of FAS may be critical to the life cycle of the virus (Yang et al., 2008). The information gained from the research presented in this thesis, as well as the current knowledge on lipid metabolism dysfunction in HCV infection, will help to develop new treatments for HCV infection (Amemiya et al., 2008; Fujita et al., 2006; Bader et al., 2008).

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